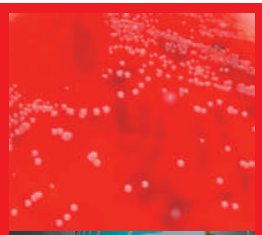

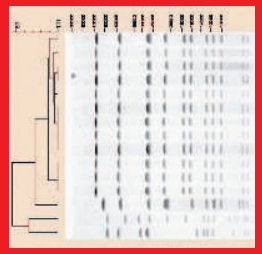

RECENT ADVANCES IN RABBIT SCIENCES

Edited by L. Maertens and P. Coudert

This book is the result of the COST 848 cooperation Action between rabbit scientists of 14 different countries. The purpose to cover the latest advances in rabbit science has been achieved in this comprehensive work. The different reviews were written by experts in their field and are grouped in 5 chapters: reproduction, housing and welfare, pathology, nutrition and feeding strategies and finally meat quality and safety. **An overview of nearly all relevant research related to rabbit production can now be found in one cover.**

Compared to other animal productions, rabbit research has received relatively little attention. Nevertheless the rabbit can be used as an economical and easy to manipulate model for other animal productions. Besides some species proper characteristics, the reproduction, pathology or digestive physiology research executed in rabbits could also be valuable for researchers working with other species.

Rabbits are important providers of meat in many EC countries. The species is known for its high reproduction capacity, rapid growth rate and the possibility of utilizing high fibre containing raw materials. Moreover, rabbit meat is appreciated for its healthy image e.g. low ω6/ω3 ratio. Rabbits are highly adaptable to be reared under different production systems and consequently also of considerable value both for small scale production and in developing countries.

An important chapter has also been devoted to the housing and welfare of rabbits. As a caged animal, pressure is executed to improve the housing conditions. The behaviour in different conditions is extensively reviewed and first attempts to enrich the environment of this social animal are given consideration.

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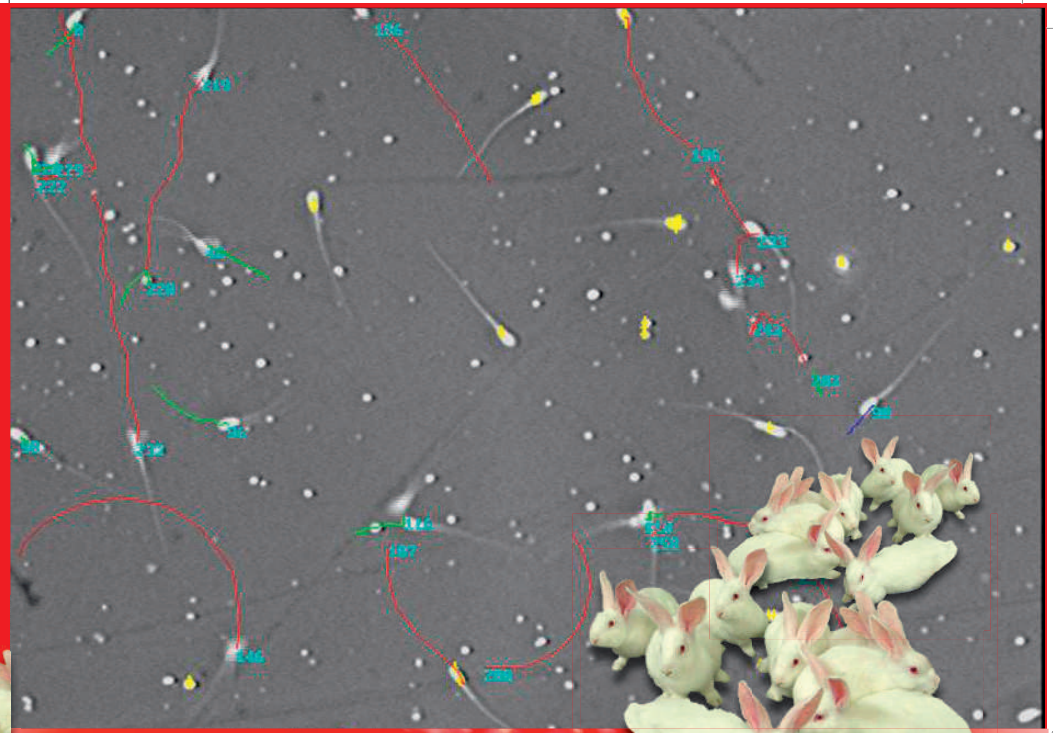

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Recent Advances in Rabbit Sciences

Edited by

L. Maertens

Institute for Agricultural and Fisheries Research

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Melle, Belgium

and

P. Coudert

INRA

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Preface

When some colleagues, most of them now retired as I am too, and I organized the First World Rabbit Congress in Dijon (1976), fairly all rabbit scientists worked in their laboratories with very few contact with rabbit scientists in other countries. Most of them worked in a national disciplinary unit devoted e.g. to reproduction, nutrition, genetics or pathology, and they had more contact with specialists in these disciplines working on other animals such as pig, poultry, dairy or beef cattle, than with rabbit specialists.

By the end of the 19th century, rabbits were mainly considered as laboratory animals in research and in 1976 it was a new idea to consider the rabbit itself as the main subject of the studies. At the same time in Europe (mainly southern and eastern Europe) the structure of rabbit production also changed. Rabbit breeding became a true agricultural activity with the same status as beef cattle or poultry production, and simultaneously in the country farms, the backyards with some laying hens, some rabbits and some ducks or chickens disappeared progressively.

To valorize the well-known rabbit ability to breed, it was necessary to know better all the reproduction mechanisms but also to feed does and their litters correctly, to house all these rabbits in adequate cages respecting their basic needs for comfort (the welfare concept was not yet widespread), to quantify the possibilities of genetic transmission of characters of economic interest, to know the effects of environment on rabbits' pathology and/or to the sensibility to specific agents. In short there was a great need for interdisciplinary studies and simultaneously for disciplinary studies on rabbits considered as individuals or groups.

Year after year, congress after congress, symposium after symposium, a significant increase of inter-laboratory and interdisciplinary cooperation and publications was noticed. Progressively the scientist's image has changed from *e.g.* a nutritionist working with rabbits into a specialist in rabbit nutrition. The target of the studies was no more the nutrition mechanisms with rabbits used as models, but the rabbit's nutrition with the objective to valorize the great biological possibilities of this species. In addition, it must be underlined that the studies on rabbits are done now with the idea of complementarity: each discipline needs the help of the others to construct a coherent scientific basis for practical use.

Because I am one of those who have worked to encourage this evolution during the last 30 years, I am very proud to preface this book which is the concretization of interdisciplinary work of which the rabbit is the target. Two thirds of the articles are signed by scientists from 2 or 3 and even more countries, after a common work involving an impressively greater number of European people and countries who worked together for 6 years. I hardly expect that the mass of high quality scientific content of this book will be sufficient to convince the European backer to renew the very positive results of the COST 848 Action. May I call upon the EC for support to continue the collaboration between rabbit scientists; the result of such cooperation will be much greater than the financial input.

François LEBAS
First President of the World Rabbit Science Association (1976-1980)
Rabbit scientist

Introduction

Research in small areas or groups suffers of limited possibilities characterized in many cases by fragmented, short term and discontinuous projects. Moreover research teams are mainly focused on one area lacking a multidisciplinary approach. The field of rabbit research was such an example and motivated us to try to use the COST (European Cooperation in the field of Scientific and Technical Research) framework to overcome this problem. COST is an intergovernmental European framework for international cooperation between nationally funded research activities. COST creates scientific networks and enables scientists to collaborate in a wide spectrum of activities in research and technology.

When in 2000, COST 848 action was approved an excellent platform was born for a multidisciplinary approach of rabbit research. Thirteen countries (Austria, Belgium, Czech Republic, France, Germany, Greece, Hungary, Italy, The Netherlands, Poland, Portugal, Slovenia and Spain) signed the memorandum of Understanding and later on also Switzerland did.

The research was split up into 5 working groups. Immediately 5 outstanding researchers were willing to manage a workgroup. During the 5 years duration of the COST 848 Action, they fulfilled this task with enthusiasm and contributed largely to the success of the Action. Therefore, we are very grateful to Prof. C. Boiti (University of Perugia), Prof. S. Hoy (University of Giessen), Dr. D. Licois (INRA-Nouzilly), Dr. T. Gidenne (INRA-Toulouse) and Prof. Blasco (University of Valencia). Moreover they accepted to coordinate a chapter of this book.

During the Action, 3 different Scientific Officers administered COST 848: Dr. R. Mulder, Dr. J. Williams and Dr. B. Stol, respectively. As chair I had very positive experiences with the collaboration with them, although sometimes they were very restricted in their financial possibilities due to the limited COST budget available in 2003. All of them were very helpful and tried to minimize the bureaucracy.

In all, over 170 researchers from 51 Universities of Institutes have participated in the Action. Many collaborations, exchanges and fruitful discussions were established. A wide range of results was presented during the 14 workgroup meetings and the 8 small meetings. Moreover, due to the COST 848 action, 22 COST funded short term scientific missions (from 1 week till 2 months) have taken place and were particularly useful to young scientists learning new techniques in specialized laboratories.

In many areas significant progress has been made during the Action, leading to the publication of the proceedings of the workshops but also to many publications in scientific journals. However, as a final dissemination of the Action, we have chosen for the publication of a book with all relevant achievements. Such a review with an up to date knowledge of the different areas in rabbit research was lacking. The title of the book reflects the advances grouped in 5 chapters corresponding with the 5 working groups of COST 848. The different coordinators of the chapters have invited leading scientists to review the latest advances

Although this book is focused mainly on intensive rabbit meat production it should not be forgotten that the rabbit is also used for wool production or that it is increasingly popular as a companion rabbit. The research reviewed could also be of interest to those involved in such use. Moreover, the rabbit has shown to have enormous potential in developing countries both in backyard production and in larger production units. We hope that this book is an instrument too for researchers in those parts of the world.

Finally it is a great pleasure to acknowledge COST, the COST Office, meeting organizers, contributors but also all the colleagues who participated in the Action. The warm contacts and pleasant stays in many parts of Europe have contributed to a large extent to genuine exchanges and collaborations and have significantly increased the level of scientific research in this domain.

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Chapter 1

REPRODUCTION

Coordinated by Cristiano BOITI

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Throughout Europe, efficient reproductive methods, based on sustainable breeding systems and artificial insemination (AI) technique, are necessary to guarantee the development of commercial rabbit production towards those high standards for quality and food safety as required by an increasing number of health-oriented consumers. Too often, however, reproductive efficiency is now achieved by means of exogenous hormonal treatments at the expense of a high culling rate and inadequate welfare conditions.

In the framework of COST Action 848, these topics were addressed by a group of dedicated scientists gathered around the WG-1. This group, including researchers with such different backgrounds as from animal physiology to applied reproduction, provided a unique environment capable of fostering new ideas in the field of rabbit reproduction. In fact, both basic and applied research are deeply required for improving the knowledge-basis of the many aspects that still remain fussy in order to better cope with problems associated with the treatment of sub-fertility of does and to adopt new management strategies better tailored to rabbit sustainable production and welfare. Although artificial insemination is largely employed, several aspects regarding the fertility of bucks and the quality of semen and its conservation also need to be investigated.

Discussion after discussion during several meetings, by taking advantage of the multi-disciplinary approach attitude of the WG1 components, the WG1 ended in 2005 with the publication of guidelines for applied reproduction trials with does and bucks in the "World Rabbit Science" journal, and with the 4 contributions integrated in Chapter 1 of the present book.

In sub chapter 1.1, several physio-pathological aspects related to reproduction disorders of does are analyzed under new angles emerging from recent progress in our understanding of the mechanisms involved in reproductive functions. New perspectives in rearing systems of rabbit does aimed at reducing the high culling rate of young does, due to early death, diseases, and reproductive problems, are examined by Rommers et al. in sub chapter 1.2. Alternative methods for oestrous synchronization of lactating does are thoroughly explored by pointing out the balance between pros and cons of each technique in terms of efficacy and usefulness, compared to the hormonal-based methods for the preparation of does to AI. Finally, the subchapter of Castellini and colleagues deals with a theme of growing interest and importance, providing up-to-date information on rabbit semen as well as on management of bucks raised for this scope.

Taken together, this chapter not only encompasses the most intriguing and hottest issues currently debated in rabbit reproduction, but also projects an outlook on the future that should be very useful for both scientists and farmers. Should this latter goal be achieved, if only partially, our efforts would be fully rewarded.

1.1. Reproductive physiopathology of the rabbit doe

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1. Introduction

Full reproductive success, culminating in the delivery of viable foetuses followed by nursing of offspring, necessarily requires tight co-ordination and the fine-tuning of various sequential processes encompassing follicular development, ovulation, fertilisation, embryogenesis, embryo implantation and gestation. The entire sequence is under hormonal control, but interactions with the other two main regulatory systems of the organism, the nervous and immune systems, also occur as exemplified by the neurocrine nature of the hypothalamic hormones subserving the pituitary gland and by the dual role of the prostaglandin (PG) F2α (PGF2α) and PGE2, acting either as hormones or mediators of the inflammatory response.

Our knowledge of the physiological aspects that control the reproductive function of the female rabbit doe is rapidly expanding (Boiti, 2004). Today, the overall picture of the endocrinological pathways that modulate the function of the hypothalamic pituitary ovarian (HPO) axis is relatively well known and provides a working basis for studying the physiology of reproductive disorders associated with stress, malnutrition, infection and ageing. However, only the main components of the female sexual function will be discussed in this chapter, with an emphasis on those reproductive disorders best characterised as having an impact on the productive efficiency of rabbits (Castellini and Boiti, 1999).

2. Hypothalamic-pituitary-ovarian axis

Several hormones produced by the hypothalamus, pituitary, and ovary come into play in a precise and co-ordinated fashion to control folliculogenesis, the oestrous cycle and sexual behaviour, and ovulation via a series of complex feedback mechanisms.

2.1. Folliculogenesis

The formation of rabbit oocytes includes several steps, which basically occur in most domestic animals: 1) Generation of primordial germ cells; 2) migration of primordial germ cells to the respective gonads; 3) colonisation of the gonads by primordial germ cells; 4) differentiation of primordial germ cells to oogonia; 5) proliferation of oogonia; 6) initiation of meiosis, and 7) arrest at the diplotene stage of prophase I of meiosis (Van den Hurk and Zhao, 2005). In rabbits, oogenesis is completed during the first 2 weeks of neonatal life simultaneously with growth of the primordial follicles (Gondos, 1969). However, the majority of oocytes degenerate during their initial meiotic activities. The 4th to 8th week-old rabbit ovaries already contain follicles at progressively more mature stages of early development (Lee and Dunbar, 1993; Lee et al., 1996).

Aggregates of membranes channels form gap junctions, which allow communication among granulosa cells and between granulosa cells and oocytes throughout follicular development, oocyte maturation and ovulation (Eppig, 2001). Only oocytes surrounded by intact cumulus cells can positively respond to hormonal signals, such as steroids and growth factors (Lorenzo et al., 1997), resulting in progressive follicular development, oocyte growth and maturation. The rabbit is known to generate polyovular follicles. Most of the developing follicles contain 2 to 3 oocytes, which develop according to their intrafollicular position. Peripheral oocytes have less chance of resuming

meiosis in comparison to centrally localised oocytes. Consequently, it is unlikely that all the oocytes in one follicle get fertilised (Al-Mufti et al., 1988).

Waves of follicles continuously develop to the antral stage under the tonic action of FSH (Fleming et al., 1984) and regress at approximately 7-10 day intervals. It has been demonstrated that gonadotropin applied for superovulatory purposes resulted in a higher number of embryos when donor animals were pre-treated with progesterone for 15 days compared to embryos recovered from solely FSH injected does (Besenfelder et al., 2002).

Rabbit embryos are surrounded by an extraembryonal glycoprotein-matrix, the zona pellucida, and additionally by oviductal secretions, known as the mucin layer, which are removed shortly before attachment to the endometrium (Betteridge, 1995). The synthesis and assembly of the zona pellucida occurs during the initial stages of folliculogenesis. It has been suggested that both the oocyte and the granulosa cells synthesise zona pellucida proteins (Grootenhuis et al., 1996; Lee and Dunbar, 1993). Although the extracellular matrix consists of three major glycoproteins, the post translational modifications result in a heterogeneity, which is responsible for specific mechanisms such as sperm-egg interaction, oocyte and embryo protection, immune response, selective metabolic barriers and physio-pathological pathways (Prasad et al., 2000). Attention is chiefly paid to the zona pellucida during early embryonic development, since at this stage all the signals of the embryo-maternal dialogue have to pass through this matrix. The matrix modulates, belatedly deliberates, prevents or facilitates signal propagation and attracts this compact structure as a valuable tool for investigating captured residues of early embryo-maternal signalling (Herrler et al., 2002).

2.2. Oestrous cycle and oestrus behaviour

Rabbits do not have a well-defined oestrous cycle and they are often, erroneously, considered to be permanently in oestrous. Sexual receptivity can be evaluated more accurately by the behavioural test in the presence of a vasectomised buck rather then by the colour of the vulva and its turgidity (IRRG Guidelines, 2005). The relationships between female sexual behaviour, sex steroid hormones, and colour and turgidity of the vulva have been investigated in rabbits throughout pregnancy, pseudopregnancy, and the post partum (Stouffet and Caillol, 1988; Rodriguez and Ubilla, 1988). During pregnancy, no clear correlation was detected; moreover, the does were sexually receptive despite high blood progesterone and low oestrogen concentrations. In contrast, during pseudopregnancy, females with a white vulva never accepted mating and the number of those with a red vulva, being sexually receptive, gradually rose toward the end of pseudopregnancy when progesterone declined to basal levels. In the post partum period, the does are highly receptive the first day after parturition and shortly (1-2 days) after weaning (Theau-Clément, 2000). However, great variability exists among individual rabbit females depending on parity, lactation stage, and other factors (Theau-Clément and Roustan, 1992).

Whereas the effect of plasma progesterone on oestrus behaviour depends on whether the does are either pregnant or pseudopregnant, administration of progesterone to estradiol-treated rabbits consistently suppresses sexual receptivity. In pseudopregnant not-receptive females, estrogens were not detectable in peripheral serum, but ranged between 15-140 pg/ml in those classified as receptive (Caillol et al., 1983). However, the physiological role of sex steroid hormones and their receptors in modulating genital hemodynamics (responsible for the external appearance of the vulva in terms of colour and turgidity, smooth muscle contractility of the genital tract, neurotransmitter receptor expression in the hypothalamic-pituitary axis, and, finally, sexual behaviour) still requires further investigation in rabbits. Surprisingly, the role of androgens in the oestrous behaviour of rabbits has not been explored, despite the fact that a substantial amount of estrogens may derive from peripheral conversion of adrenal androgens and from aromatisation of testosterone in the brain (Roselli et al., 1997).

Results indicate that sexual receptive behaviour of the doe is related to the presence of more large follicles on the ovary (Kermabon et al., 1994) and higher plasma concentration of estrogens (Rebollar et al., 1992). It is now well established that sexual receptivity of the doe at the time of artificial insemination (AI) greatly affects fertility (Theau-Clément and Roustan, 1992; Castellini and Lattaioli, 1999) and its components, ovulation frequency (Rodriguez and Ubilla, 1988), fertilization rate (Theau-Clément, 2001) as well as prolificacy resulting from ovulation rate, embryo and foetal survival (Theau-Clément and Roustan, 1992, Castellini and Lattaioli, 1999 Theau-Clément, 2001). Consequently, the productivity of receptive does is higher than that of non-receptive does (primiparous: 6.3 *vs.* 1.6 weaned rabbits/AI, multiparous: 7.8 *vs.* 2.9 weaned rabbits/AI, Theau-Clément, 2001). Morever, regarding the physiological status of does at the time of AI, lactating, non-receptive does have the worst reproductive performance following AI.

2.2.1. Hormonal protocols for oestrous induction

The problem of oestrous synchronisation in the post-partum period is of crucial importance for the successful employment of the AI technique. It is therefore not surprising that several hormonal protocols have been devised for inducing sexual receptivity and synchronising oestrus in rabbit does at the time of insemination. The non-hormonal protocols for oestrous induction are examined in another chapter of this book.

2.2.1.1. Pregnant mare serum gonadotropin.

Comparable results in terms of the number of receptive does at the time of AI have been achieved on 11-day lactating does with doses of pregnant mare serum gonadotropin (PMSG or eCG) ranging from 10 IU (Bonanno et al., 1991), to 20 (Bonanno et al., 1990; Maertens, 1998), 25 (Theau-Clément and Lebas, 1996, Theau-Clément et al., 1998a), 30 (Mirabito et al., 1994b), and even 35-40 IU (Castellini et al., 1991; Bourdillon et al, 1992). Moreover, the positive effect is maintained after several injections during 7 (Boiti et al., 1995), 9 (Theau-Clément et Lebas, 1996) or 11 repeated cycles (Theau-Clément et al., 1998a). In contrast, it must be emphasised that, for embryo transfer programmes, higher doses of PMSG are used for superovulation purposes, but can cause over-stimulation of the ovary.

A PMSG injection before AI generally increases rabbit-doe fertility, but its efficiency could depend on the conditions of treatment (dosage, method of injection, interval between injection and AI) and the physiological status of the does. However, Alabiso et al. (1994) did not improve fertility with 40 IU, compared with 20 IU of PMSG.

The optimal interval between the PMSG administration and AI has not been evaluated, but most studies considered a 48-hour interval as the most effective. In fact, when 20 IU of PMSG were injected 72 hours before AI, no fertility improvement was obtained. The efficiency of PMSG treatments varies according to the parity of the does. PMSG does not improve fertility of nulliparous rabbits (Castellini et al., 1991; Parez, 1992; Alabiso et al., 1994). Conversely, PMSG generally improve fertility of primiparous (Bourdillon et al., 1992; Davoust, 1994; Maertens, 1998) and multiparous lactating does (Davoust, 1994; Mirabito et al., 1994b; Theau-Clément and Lebas, 1996; Theau-Clément et al., 1998a). PMSG injections are therefore not justified for treating non-lactating does, since they already have high reproductive potential. Some authors obtained bigger litter sizes after administering PMSG, but Theau-Clément and Lebas, (1996) demonstrated that the higher prolificacy of treated does was only associated with the increase in the percentage of receptive does.

Due both to its exogenous protein nature and to its high molecular weight, repeated PMSG administration may stimulate the immune system to form antibodies against PMSG. In rabbits, the immune response to PMSG was first reported by Canali et al. (1991) and subsequently confirmed by Boiti et al. (1995) after injecting 40 and 20 IU of PMSG, respectively. According to these authors, anti-PMSG antibody titres rose after the third repeated injection concomitantly with a decline in fertility. Theau-Clément et al. (1998b) studied the evolution of anti-PMSG antibody levels using different PMSG dosages (8 or 25 IU) on 124 primiparous does during 11 series of inseminations. PMSG antibodies were found only after the 6th injection. At the end of the experiment, only 15 and 39 % of the does treated with 8 or 25 IU of PMSG, respectively, developed immunity against PMSG. Moreover, the overall productivity of the lactating does was not significantly related to PMSG immune response (Lebas et al., 1996; Theau-Clément et al., 1998b).

On the basis of current knowledge, the routine use of PMSG (20-25 IU, 48 hours before AI) on 11-day lactating does, considerably increases the percentage of receptive does at the time of insemination and consequently their fertility and productivity with no significant immune response. However, only 8 IU of PMSG are generally recommended to stimulate 4-day lactating does (Theau-Clément et al., 1998a; 1998b).

2.2.1.2. PGF2α

Besides the large spectrum of actions ascribed to PGF2α, and its analogues, either physiological or pharmacological, the main PGF2α-dependent effects useful for the control of reproduction rely on their luteolytic property. PGF2α or its analogues have also been employed with the aim of synchronising oestrous and improving the fertility rate of does artificially inseminated on either post-partum day 4 or 11, when the does are lactating. With the purpose of oestrous synchronisation, Facchin et al. (1992) used 200 µg of alfaprostol, a PGF2α analogue, either 72 or 96 hours before artificial insemination. Compared to does treated at the same time intervals with 20 IU of PMSG, the authors obtained better results in terms of fertility rate and viability of new-borns in does injected with alfaprostol 72 hours before AI. Alvarino et al. (1995) employed both natural and synthetic PGF2α injected 48 hours prior to AI in either nulliparous or multiparous does. They concluded that PGF2α increases the ovarian response and overall fertility performance of nulliparous does, even if the animals are naturally very fertile and do not need hormonal stimulation. They also found that PGF2α can substitute PMSG only in lactating does inseminated at day 11 post-partum, but not at day 4.

Other authors later reported somewhat different results, which add uncertainty to the usefulness of the systematic employment of prostaglandin for synchronising oestrus. In a recent study, Mollo et al. (2003) did not find any improvement in fertility rate and number of newborn in does receiving PGF2α analogue, alfaprostol, at day 8 postpartum, in comparison to does treated with PMSG or to untreated does.

A simultaneous treatment of PMSG and PGF2α analogue, alfaprostol, has been proposed by Facchin et al. (1998). The reason for this association is grounded on the luteolytic action of PGF2α and to the FSH-like function of PMSG. In does having a

high plasma progesterone concentration (P+), the PGF2α could be effective in removing the progesterone block, although PMSG is likely ineffective. Thus, in herds having a significant incidence of P+ does (see paragraph 5.1.2), the association could be efficient by simultaneously recovering the P+ does (PFG2α action) and by stimulating those with basal progesterone (PMSG action). The efficiency of this pharmacological association, however, may be linked to the proportion of P+ does at the time of treatment and this fact, often underestimated, could explain the contradictory results obtained by different authors using a similar protocol (Stradaioli et al., 1993; Alvarino et al., 1995; Mollo et al., 2003). It is probable that several other factors not yet understood are also involved. Independently of the cause, however, it is now clear that part of the problem may be explained by the finding that a relevant, but highly variable, proportion of does may present high progesterone levels at the time of artificial insemination (see paragraph 5.1.2).

As a result of extensive embryo transfer programs in rabbits, the Besenfelder group has accumulated over time wide experience of the techniques for hormonal synchronization including super-ovulation. These protocols, involving about three thousand female rabbit, were based on hCG of different origin, eCG, FSH alone or combined with progesterone (Besenfelder et al., 2002), followed by GnRH or hCG. However, due to the high variability between and within the same hormonal scheme, as well as to individual variability between does linked to age, season, breed and others (personal data) no clear evidence has emerged on the definition of a standard procedure. Independently of treatments, in fact, the percentage of ovulating does after GnRH ranged between 80-100%, whereas the ovulatory rate (mean number of ova for each ovulating doe) varied from 10 to 30. These data suggest that the pre-ovulatory condition greatly affects ovarian response to synchronization and super-ovulation treatments, and that the post-ovulatory loss of oocytes, within the 4 to 6 hours after ovulation, may reach 20%. Other variable losses may occur as a consequence of unfertilisation, early embryo and foetal deaths, and abortion. Taken together, all these losses may well account for the common 70% average reproductive performance reported by farmers.

2.3. Hypothalamic-pituitary control of ovulation

Several different releasing (and inhibiting) factors have been identified as hypothalamic hormones. These hormones are released by different neuronal cell types into the median eminence and transmitted to the anterior pituitary via the portal vessels. Those of primary importance for reproduction involve GnRH (gonadotropin releasing hormone), which controls the secretion of pituitary gonadotropins, particularly luteinizing hormone (LH), but also follicle stimulating hormone (FSH). Mating activates diverse sensory areas, whose evoked signals funnel, via neural pathways along the spinal cord, in the brainstem and hypothalamus (Lin and Ramirez, 1991). Evidence, derived from direct sampling of portal blood from the pituitary stalk of rabbits, proved that GnRH rises rapidly after coital stimulus and peaks within 1-2 hours. The GnRH was found to precede the LH surge, whose maximal release is attained 60-90 minutes after coitus to gradually decline within the following 4-6 hours (Dufy Barbe et al., 1978).

The neural connections between natural coital stimulation and GnRH release primarily involve norepinephrine (NE) and acetylcholine neurotransmitters, since the administration of an antagonist against both messengers blocks or attenuates the ovulation process (Centeno et al., 2004). Mating induces NE release from the mediobasal hypothalamus before or simultaneously with GnRH as well as NE gene expression in neuronal cells located in the brainstem (Spies et al., 1997). Thus, the brainstem is a likely extra-hypothalamic site where coital stimuli are integrated and converted into preovulatory signals for the GnRH surge to develop.

Besides NE, a variety of different transmitter systems (Kaynard et al., 1990), including, neuropeptide Y (NPY), galanina, α-endorphin, interleukin-1 (IL-1), corticotropin-releasing hormone (CRH), nitric oxide (NO), and GABA are likely implicated in the regulation of LH secretion in animals by generating, maintaining and/or modulating the GnRH surge process (Gonzalez et al., 1993; Pau and Spies, 1997). These inputs target at different hypothalamic loci both local pre-motor neurones, and GnRH neurones to alter the GnRH pulse pattern, but none of these have been studied in the rabbit.

The influence of gonadal hormones on the phasic release of gonadotropins varies greatly among species. It has been well established that combinations of estrogens, androgens and progestagens exert positive or synergistic effects on certain target organs, e.g. the hypothalamic-pituitary axis, and antagonistic or complementary effects on others, e.g. on the endometrium. During the preovulatory period, in spontaneous-ovulating species, the GnRH surge released is triggered by increasing levels of circulating estradiol-17α. In contrast, in rabbits as well as in other induced-ovulating species, the reflex GnRH surge-generator neuronal network within the hypothalamus is usually unresponsive to the positive feedback by oestrogen (Dufy-Barbe et al., 1978).

In addition, estradiol receptors (ER) have not been found in GnRH secreting neurons. However, the localisation of ER in neuronal cells of the

infundibular nucleus (IN) of the hypothalamus suggests that they are related to gonadotropin control, since GnRH-containing neurones have been found in the IN of rabbits (Foster and Younglay, 1991.

Recently Caba et al., (2003a; 2003b) described the neuroanatomical distribution of receptors for estradiol and progesterone (PR) and also their regulations and functions in the female rabbit forebrain. They showed that neurones, belonging to restricted regions of the female rabbit forebrain, including IN, preoptic, and paraventricular areas, express abundant PR and ER, which are either sensitive or insensitive to the down-regulatory effects of estrogens and progesterone. Expression of ER in both hypothalamus and anterior pituitary was found to be down regulated by a negative energy balance occurring after only two consecutive days of fasting (Brecchia et al., 2004).

The neuronal limb of the ovulatory reflex can be bypassed by the exogenous administration of GnRH (or its analogues) that act on the anterior pituitary to release LH within a few minutes after binding to GnRH receptors in basophils cells (Rispoli and Nett, 2005). The profile of the LH peak surge obtained by i.m. administration of the GnRH analogue, buserelin, closely matches that found in mated females following coital stimulation. The GnRH agonist also has a direct effect on the ovary and in fact can induce ovulation in hypophysectomised animals and in a vitro perfused system (Koss and LeMaire, 1985).

The availability of inexpensive hormonal products for the induction of ovulation also largely contributed to the successful spreading of the AI technique in rabbits. To induce ovulation, buserelin could also be added to the seminal dose for intravaginal administration (Quintela et al., 2004). Ovulation can also be achieved by injection of human corionic gonadotropin (hCG), which bypasses the hypothalamus-pituitary axis to directly target the ovarian follicles, but due to immunological risks, its use is now very limited.

2.3.1. Biochemical events of ovulation

Soon after mating as well as after GnRH injection, the ovulation process is initiated by LH and FSH acting together. Both hormones are secreted by pituitary gonadotropes cells in response to GnRH, although with different patterns. The LH surge may lead to a 100-fold increase in blood levels within 60-90 minutes after coital stimulation or GnRH challenge, whereas FSH secretion is more blunted with a characteristic increase 24 hours later. This delayed surge of FSH is likely responsible for the recruitment and development of a new ovarian follicle population which may provide the growing corpora lutea (CL), originated by recently ovulated follicles, the necessary trophic support of estrogen.

Ovulation depends upon the LH surge and occurs 9-10 hours later. During this interval, several maturation changes take place in the Graafian follicles and ova: follicular hyperaemia and swelling, expansion of the cumulus complex, and the shedding of ova into the fallopian tubes. Ovulation involves only those mature ovarian follicles that have built up an adequate number of receptors for LH under the tonic action of FSH and estrogens. Following binding of LH to its own dedicated receptor, cyclic AMP mediates the LH-induced prostaglandin synthesis in ovulatory follicles as well as the downstream cascade of PKA-dependent events that lead to luteinisation of granulosa and thecal cells. Prostaglandins are synthesised from arachidonic acid derived from membrane phospholipids under the action of specific phospholipases. It is now accepted that the ovulatory surge of LH induces an inflammatory-like reaction in mature ovarian follicles, which causes the rupture of the ovarian surface epithelium (Espey et al., 1986). Synthesis of prostaglandins, particularly PGF2α and PGE2, histamine, bradykinin, and other mediators are necessary for the ovulation to occur since indomethacin, antihistamines, and other specific blockers inhibit ovulation in both *in vivo* rabbits and *in vitro* perfused rabbit ovary (Kobayashi et al., 1983).

LH induces a sharp increase in the secretion of several hormones such as estradiol-17α, progesterone, 20α-hydroxyprogesterone (20α-OHP), and testosterone (Hilliard et al., 1974), all of which reach high values in the circulating blood only a few minutes after GnRH treatment or mating. The physiological relevance of the rise in the level of these hormones is still under debate, since they may affect both the hypothalamus and the pituitary as well as other ovarian components. Interestingly, among these steroids, the 20α-OHP is the main progestin produced by the ovary in quantities ten-fold higher than progesterone. 20α-OHP is synthesised by the interstitial tissue of the ovary which is quite abundant in the rabbit ovary. However, although 20α-OHP can be regarded as an independent hormone and not a metabolite of progesterone, its function still remains uncertain.

3. Ovarian components

3.1. Ovarian follicles

During folliculogenesis, primordial follicles made up of a single layer of granulosa cells, develop to form secondary follicles in which the outer theca layer deploys a vascular network and the granulosa cells increase in number and layers. Growth to mature cycling type follicles (antral follicles) is associated with a gradual enlargement and proliferation of the theca capillaries and with capillary hyper-permeabilisation in ovulatory and post-ovulatory follicles, after hCG stimulation (Macchiarelli et al., 1995). The co-existence of a

large number of follicles at different stages of development within the ovary requires mechanisms that permit their selection under similar endocrine stimulation. It is probable that this task is assured by the paracrine/autrocrine actions of several local hormones and factors that play a key role in the local responses of follicles and the integration with other discrete ovarian components. The granulosa cells lining the follicles actively synthesise estrogens via aromatisation of testosterone provided by the surrounding theca cells.

3.2. The corpus luteum

The corpus luteum (CL) is a transient endocrine gland that secretes progesterone to support pregnancy. In rabbits, the CL is maintained throughout gestation, a characteristic that differentiates the rabbit from other species. The CL are formed from ovulated follicles in a process that involves angiogenesis and tissue remodelling under the influence of several endothelial-derived factors, including vascular endothelial growth factor (VEGF), transforming growth factor (TGF-α), and fibroblast growth factors (FGFs) acting locally in a paracrine/autocrine manner (Schams and Berisha, 2004), together with different luteotrophic hormones such as LH, estradiol-17α (Webb et al., 2002) and probably also PGE2. Within the CL, the function of each cell type, such as large and small luteal cells, endothelial cells, fibroblasts, and immune cells, macrophages and lymphocytes, are regulated by various local factors, including lymphokines, growth factors, prostaglandins, and a large array of hormones. The overall balance between luteotrophic and luteolytic activities, however, may change with the relative age of the CL.

3.2.1. Luteolysis

The luteolytic mechanism plays a key role in reproductive physiology, given that it controls the length of the oestrous cycle in spontaneous-ovulating species. This mechanism is also important in rabbits. In fact, if fertilization does not occur or implantation is unsuccessful as well as at the end of gestation, luteal regression will wipe out unnecessary CL and remove the block by progesterone. In rabbits, luteal regression normally begins on day 14 of pseudopregnancy and is completed around day 18 when progesterone declines to basal value (Browning et al., 1980).

Luteolysis is a streamlined process (Niswender et al., 2000) that involves functional and structural changes ending with complete demise of CL through apoptotic pathways. Although cell deletion via apoptosis is the final outcome in regressing CL (Nicosia et al., 1995), the actual machinery and the timing of its induction in rabbits, as well as the subtle interplay with locally produced growth factors, cytokines, luteolytic and luteotrophic hormones, exhibiting both pro- and anti-apoptotic properties, are still unclear. Interestingly, depending on the luteal stage, we have found an opposite response in the expression of the p53 gene transcript during PGF2α-induced luteolysis. The protein p53 may act as both a tumor-suppressor and transcription factor that, upon activation by DNA damage and other cellular stress signals, leads to the transcription of genes triggering cell-cycle arrest, apoptosis, or DNA repair (Levine 1977; Lakin and Jackson, 1999).

3.2.1.1. Role of PGF2α in luteolysis

In rabbits, as in many others species, CL regression is driven by PGF2α, which has been identified as the main luteolytic factor of uterine origin (Lytton and Poyser, 1982). In fact, prolonged luteal function has been observed in hysterectomised (Scott and Rennie, 1970) as well as in intact rabbits with induced endometritis (Boiti et al., 1999), thus indirectly confirming the importance of the endometrium for properly timed spontaneous luteolysis.

The PGF2α–induced luteolytic model has been often employed to study the physiological mechanisms of luteal regression, because it simplifies the analysis of the time-dependent events by synchronising the initiating stimulus. The responses of rabbit CL to PGF2α during different stages of pseudopregnancy has been investigated both *in vivo* and *in vitro* (Boiti et al., 1998; Gobbetti et al., 1999; Boiti et al., 2001), and a time-dependent responsiveness to PGFα treatment was shown. Interestingly, in the early luteal phase, up to day 4 of pseudopregnancy, the growing CL are totally refractory to exogenous PGF2α administration, even if they express luteal receptors for PGF2α (Boiti et al., 2001). Thus, the stage of CL greatly influences the luteolytic response to PGF2α of either endogenous or exogenous origin, for reasons not yet fully elucidated, but probably linked to the presence of several growth and mitogen factors having luteotrophic, anti-luteolytic, or luteal protective actions.

The search to unravel the underlying mechanisms that control the life span of CL in rabbits and in other species still continues in many laboratories using different experimental approaches. The down-streaming mechanisms activated by exogenous prostaglandin PGF2α treatments in rabbits has received great attention in the past few years by our laboratory (Boiti et al., 2000; Boiti et al., 2002; Boiti et al., 2003; Boiti et al., 2005b, 2006). PGF2α after engagement to its specific binding sites (FP), activates the G protein-dependent PLC/PKC pathways, causing breakdown of phosphatidylinositol to generate DAG and IP_3, increase intracellular stores of Ca^{2+} and phosphorilation of transcriptional factors (Fig. 1). As a consequence, depending on the luteal stage – early, mid, or late – several genes are either up or down regulated, thus modifying the biological responses of the different luteal cell types and driving the final

outcome of CL toward functional survival or death. In our laboratory, by using CL explanted *ex vivo* following PGF2α bolus challenge at different luteal stages, we have characterized the dynamic expression of several genes coding endothelial derived factors, such as endothelin-1 (ET-1) and its two receptors subtypes (ET_AR and ET_BR), angiotensin converting enzyme (ACE), VEGF, different interleukins (IL) such as IL-1, IL-2, IL-6, chemoattractant factor for monocytes (MCP-1), and enzymes having a critical role for the synthesis of PGs, such as the inducible form of cyclooxygenase (COX-2), and for the production of nitric oxide (NO), such as NO synthase (NOS).

It is now evident that PGF2α administration to rabbits induces a local synthesis of PGF2α itself by the CL via gene up regulation and activation of COX-2 (Zerani et al., 2006a, b). This intraluteal autocatalytic mechanism, however, may have a physiological relevance only during spontaneous luteolysis. In fact, the luteolytic process triggered by the administration of PGF2α analogue is neither blocked nor blunted by pre-treatment with COX inhibitor, indomethacin (Boiti et al., 2006).

While exogenous PGF2α administration has been routinely used for many years to control the breeding of farm-animals by exploiting its luteolytic properties, in rabbits the employment of PGF2α is much more limited. The reason is basically due to the different oestrus cycles of rabbits. Therefore, while in domestic farm-animals, PGF2α-induced CL regression may be used to precisely control the oestrous cycle and the time setting for ovulation (oestrous synchronisation), in rabbits, this luteolytic mechanism comes into play only in the case of pseudopregnancy or pregnancy.

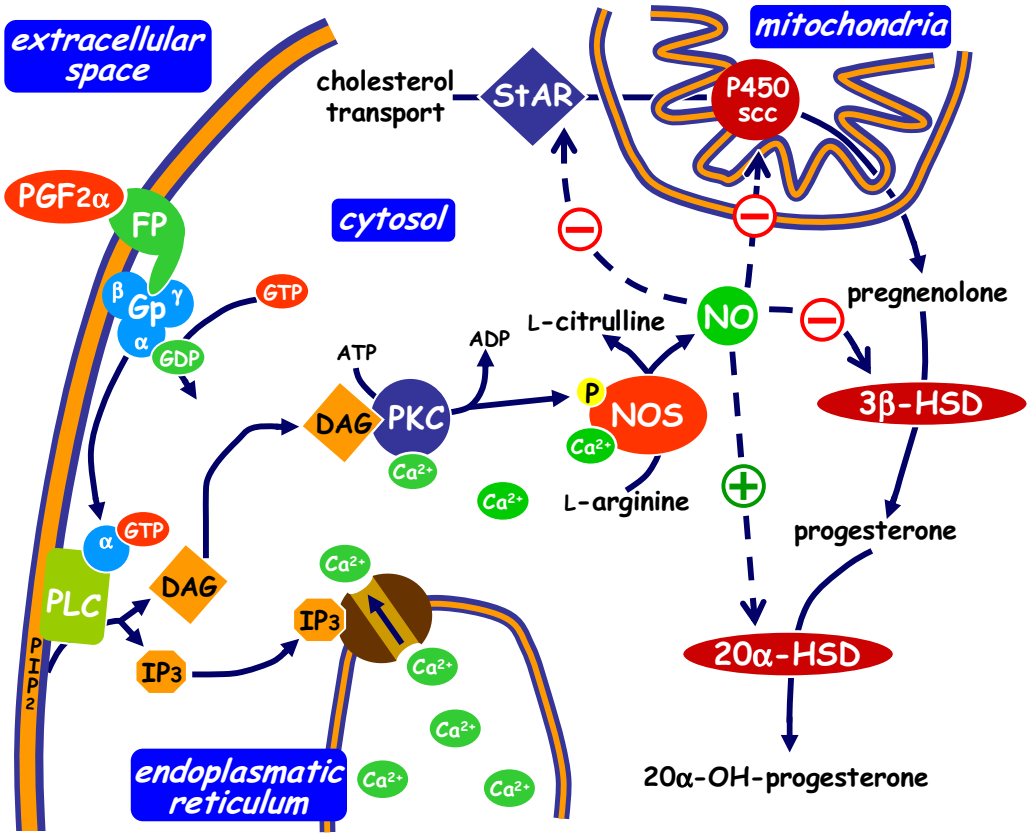

Figure 1. *Simplified model showing the intracellular pathway activated by PGF2α within a rabbit luteal cell causing progesterone down-regulation at day 9 of pseudopregnancy. The hatched lines represent the possible NOS/NO target(s). 3β-HSD: 3β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase, ADP: adenosine diphosphate; ATP: adenosine triphosphate; DAG: diacylglycerol; FP: prostaglandin F2α receptor; GDP: guanosine diphosphate; Gp: G protein; GTP: guanosine triphosphate; IP3: inositol triphosphate; NO: nitric oxide; NOS: nitric oxide synthase; P: phosphate; P450scc: cytochrome P450 side-chain cleavege; PGF2α: prostaglandin F2α; PIP2: phosphatidyl inositol diphosphate; PKC: protein kinase C; PLC: phospholipase C; StAR: steroidogenic acute regulatory protein.*

3.2.1.2. Role of ET-1 in luteolysis

Several distinct intraluteal pathways have emerged as potential candidate mediators of the PGF2α-dependent luteolytic effect (Webb et al., 2002). Prominent among them is ET-1, a potent vasoconstrictor synthesized by vascular endothelium (Meidan et al., 1999), suggesting a strict interplay between endothelial and luteal cells in the control of luteal function (Diaz et al.,2002; Ohtani et al., 2004, Acosta et al., 2004). The presence of receptors for ET-1 in the vascular components of rabbit CL and luteal cells suggests that the ET-1 system is involved in the regulation of ovarian blood flow and in steroidogenesis as well (Boiti et al., 2005a). However, by *in vivo* and *in vitro* studies, using the pseudopregnant rabbit model, we provided evidence indicating that whereas ET-1-induced functional luteolysis is linked to the prostanoid pathway and increased NOS activity as well as to the renin-angiotensin system, PGF2α does not require ET-1 or angiotensin-II to elicit its luteolytic action (Boiti et al., 2005c). In addition, a strong positive and reciprocal feedback system between PGF2α and ET-1 has also been found in rabbits (Boiti et al., 2006) similarly to that described in ruminants (Arosh et al., 2004). The intracellular mechanisms implicated in the antisteroidogenic action of ET-1 have been examined in the rabbit CL for the first time. ET-1, after engagement to its specific binding sites, it is probable that ET_AR, activates the G protein-dependent PLC/PKC pathways. Thus, ET-1, either derived by *de novo* synthesis within luteal tissue during spontaneous luteal regression or directly from exogenous administration, may use the same intracellular signaling pathway activated by PGF2α (Boiti et al., 2005a, 2006). These findings imply that in rabbit CL, having acquired a luteolytic competence, a time-sequential convergence over the same intracellular effector system by different receptor-linked signals may exist, whose downstream response pathway results in a synergistic antisteroidogenic action.

3.2.1.3. Role of interleukins in luteolysis

There is increasing evidence to support the hypothesis that luteolysis is an immune-mediated event (Tilly, 1996). In this context, several cytokines, including IL-1, IL-2, MCP-1, tumor necrosis factor □, and interferon-α, secreted by resident cells or recruited immune cells also normally found in rabbit CL (Krusche et al., 2002; Boiti et al., 2004), are probably involved as local inflammatory mediators in the processes of luteal demise (Pate and Keyes, 2001) by up-regulating gene expression for pro-apoptotic p53 and NOS as well as its activity (Boiti et al., 2004).

There is little doubt that complex interplays between different cytokines and lymphokines due to their multiple and redundant actions, growth factors, prostaglandins, and a large array of hormones are at work in the CL during luteal regression. Moreover, leptin, a 16 kDa cytokine primarily secreted by adipocyte, has also been found to have an anti progesterone activity in rabbit CL, suggesting an interesting interrelationship between nutritional condition and luteal function (Zerani et al., 2004).

4. Oviduct-Uterine components

Within the uterus three major processes take place: Capacitation, implantation, and embryo development. The oviduct represents the site where maternal and paternal gametes meet and interact. The entry into the oviduct is performed from opposite directions, thus resulting in a counter-current micro-movement. After mating or insemination, spermatozoa pass the uterine horns, reach the oviduct and bind to the ciliated epithelial cells of the caudal isthmus region (Harper, 1973a,b; Overstreet and Cooper, 1975). Near the time of ovulation there are still unknown signals, which assist in sperm release from the oviductal epithelium.

The oviductal activity is characterized by its physiological tasks ensuring:

- the maintenance of sperm to bridge the time gap between ovulation and fertilisation;
- capacitation and motility hyperactivation of sperm;
- sperm reservoir (storage), control of sperm transport (reduced polyspermia) for an optimised oocyte fertilisation (high fertilisation rate);
- early embryo development;
- coordination of embryo migration (delay until the uterus is able to accomplish further embryo development) (Hunter, 2005; Töpfer-Petersen et al., 2002; Suarez, 2002).

The ovulation itself is dependant upon a number of complicated processes with successive steps resulting in the capture of female gametes in the oviduct. Studies with hamsters revealed that the viscoelastic content of the ovulatory follicle is extruded by constant pressure as an expanded cumulus-oocyte complex (COC) (Talbot, 1983). The cumulus acquires viscosity in response to the ovulatory stimulus by deposition of an extracellular matrix between the cumulus cells (Chen et al., 1994; Salustri et al., 1992). The deposition is associated with a tremendous expansion of the COC volume (Chen et al., 1990). Impaired expansion of COCs or removal of the extracellular matrix prevents oocyte pick-up by the oviduct (Huang et al., 1997; Mahi-Brown and Yanagimachi, 1983). In the expanded cumulus mass hyaluronan is the predominant component, which is organized in granules and filaments (Zhuo and Kimata, 2001). Adhesion between hyaluronan and a specific crown region on the tip of the cilia of the infundibulum is essential for pick-up and further transport of ovulated

complexes into the ampulla (Talbot et al., 2003; Lam et al., 2000). In this context, adhesion is facilitated by structural peculiarities of the oviductal mucosa. Scanning electron microscopy depicts a complex three-dimensional architecture of the oviductal mucosa (Hunter et al., 1991). The ampullary region is characterized by prominent and lower longitudinal folds with oblique running secondary ridges and a well organized system of pockets in the interspaces (Yániz et al., 2000). The appearance of the epithelium depends on the phase of the oestrus cycle. During oestrogen dominance, densely arranged ciliated cells and protruding secretory cells are characteristic, while in the luteal phase the ciliated cells decrease both in number and height (Abe and Oikawa, 1993). The secretory activity of the oviduct epithelium displays its maximum around ovulation (Erikson et al., 1994), resulting in an increase of the oviductal fluid current (Killian et al., 1987; Killian et al., 1989). However the cilia move the comparatively large COCs slowly, indicating a transient anchorage to the oviductal epithelium (Lam et al., 2000).

Fertilisation is performed by a mixture of active and passive transport mechanisms occurring between spermatozoa, cumulus oocyte complexes, epithelium and luminal fluid. After fertilization has occurred, the early developing embryos are passively transported to the uterus by a series of closely coordinated mechanical events where activities of cilia and smooth muscle predominate. It is well known, that myosalpinx contractions propagate randomly, producing a backward-forward egg motion over short distances. These activities are generated from different pace-maker sites. These mechanisms are thought to be responsible for the contact between the hormones and nutrients contained in the tubal fluid and the gametes and early embryos guaranteeing correct fertilisation and development rather than for continuous transportation (Muglia and Motta, 2001; Germanà et a., 2002). Once the ovum and embryo are captured, ciliary activity seems to be more important than tubal contractility in transporting the ova towards the uterine cavity (Osada et al., 1999). The lumen of the isthmus is extremely narrow and contains viscous secretions, and myosalpingeal contractions are reduced (Hunter, 2005). The three dimensional myoarchitecture of the utero-tubal junction, sphincter-like species type (in rabbits), regulates the sperm ascendance towards the ampullary region (Muglia and Motta, 2001) as well as the timed utero-tubal transmission of the embryos. Recently, receptor for leptin has been characterised in the rabbit oviduct, raising questions on the possible role of this cytokine in linking nutritional information to its critical physiological function (Zerani et al., 2005).

5. Reproductive disorders

Reproduction is a highly anabolic process and redundant mechanisms have evolved to protect the mother from potentially life-threatening conditions of either external or internal origins. It has long been known that stress challenges related to adverse environmental conditions may switch off the reproductive function in farm as well as in wild animals (Ferin, 1998).

Nutrition and lactation are two other major factors affecting reproduction. Negative energy balance, especially in young rabbit does, can result in infertility because of the high energy demands for concurrent pregnancy and lactation (Theau-Clément and Roustan, 1992; Xiccato, 1996; Fortun-Lamothe, 1998). Several convergent pieces of evidence demonstrate that maternal nutritional status may exert a great influence on the reproductive function of does, which may expand into the time period after conception, involving early embryogenesis, pregnancy and birth. The pre-conception nutritional status of does, as modified by even a short-term fasting lasting 24 hours, can have a negative influence on both fertility and sexual receptivity (Brecchia et al., 2005). In does fasted for a period of 48 hours, Brecchia et al. (2005) found that peripheral plasma oestradiol-17α had lower pulse frequency and amplitude than in rabbits fed *ad libitum*. In addition, the GnRH-dependent LH secretion was much lower in these animals compared to controls. At present, however, it is not clear what metabolic signals are specifically involved in modulating reproductive function, nor whether they act directly upon the hypothalamic-pituitary-ovarian axis or indirectly to regulate the secretion of other hormones. Lactation partially inhibits all the reproductive functions of does by depressing sexual receptivity, ovulation rate, fertility and embryo development, particularly on day 4 post partum (Theau-Clément et al., 1990; Theau-Clément and Roustan, 1992; Theau-Clément and Poujardieu, 1994, Theau-Clément et al., 2000). Nevetheless, recently, Theau-Clément and Fortun-Lamothe (2005) and Feugier et al. (2005) observed that on primiparous does fertility increases after 11 days post partum, despite the progressive mobilization of body reserves during lactation. Thus, several factors concur in adjusting the reproductive function to environmental conditions through direct and/or indirect interferences acting at different levels of the gonadal axis involving the hypothalamic centres responsible for GnRH pulse release (Pau et al., 1986) and control of sexual behaviour (Wade et al., 1986), the pituitary as well as the ovary.

It is obvious that any deviation from the normal pattern of ovulation, fertilisation, migration and development within an altered microenvironment may disturb this very susceptible and complex network consisting of several well tuned steps, leading to a higher incidence of unfertilisation and poor embryo quality. It should be pointed out that

the term “embryo quality” unfortunately includes an unmanageable and steadily increasing number of well accepted elementary major and minor factors, known to rule embryo development, but unknown in its holistic context. Beside macroscopic (organ) and microscopic (morphological properties) assessment of embryo quality, new molecular-based techniques aim at increasing a more comprehensive understanding of a “good”, active embryo. A holistic analysis of maternal (Wolf et al., 2003;Bauersachs et al, 2003, 2004) and embryonic activities (Kanka et al., 2003; Sirad et al., 2005) as well as of embryo-maternal interactions promises to have a great beneficial impact on research and commercial applications.

5.1. Ovarian disorders

Systematic studies concerning the anatomy, physiology and pathophysiology of female rabbit sexual dysfunction are limited. Several dysfunctions may affect each structural component of the ovary, including follicles, oocyte, CL and interstitial gland by altering hormone synthesis and cellular division. Due to the close anatomical and physiological connections between all ovarian components, any local deviation from normality is likely to have repercussions on nearby ovarian structures, as well as on the uterus and the hypothalamic-pituitary axis.

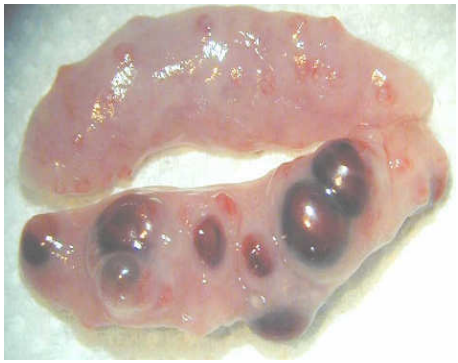

Figure 2. *Ovarian response to different gonadotropin stimulations (pFSH: total 10 mg FSH equivalent, upper ovary, and PMSG 20 IU/kg body weight, lower ovary), showing normal preovulatory follicles (upper) and several large haemorrhagic follicles (lower). In both cases, the ovaries were collected 20 hours after hCG injection for induction of ovulation.*

5.1.1. Ovarian hyperstimulation syndrome

Since the introduction of ovarian stimulation methods for embryo transfer programmes, ovarian hyperstimulation syndrome (OHSS, Fig. 2) has become a problem. OHSS is mostly iatrogenic in nature, and is associated with PMSG treatments for superovulation purposes. The overall incidence of OHSS varies greatly, depending on individual sensitivity to PMSG dosage, which, in turn, is probably associated with the presence of follicles at different stages of development and, consequently, with the specific sexual steroid milieu and relative abundance of receptors for gonadotropins. However, it is also possible, although rare, for OHSS to be found in does treated with the standard doses of PMSG recommended for oestrous induction.

Using PMSG for hormonal treatment in rabbits, at least for superovulation, quite often results in an inadequate stimulatory effect on follicular growth and oocyte maturation. The problem is that the endogenous secretion profile of FSH and LH is only roughly mimicked by PMSG, which is a very large, immunogenic glycoprotein, about 45 kDa (Combarnous et al., 1981), expressing both FSH-like and LH-like biological activities. Moreover, FSH is generally responsible for follicular growth, whereas LH exerts its effect mostly on final oocyte maturation, ovulation and luteinisation. Regarding the very high LH-activity of PMSG, it seems noteworthy that even in small growing follicles there are LH receptors which may respond too early to LH. At the end, this leads to the recruitment of an asynchronous cohort of follicles including both small as well as over-aged ones, which do not ovulate, or that ovulate without releasing oocytes suitable for fertilisation and further development. Embryo production between PMSG and FSH treated animals is sigbnificantly different (Allen, 2001; Licht et al., 1979; Monniaux et al., 1997; Besenfelder et al., 2002; Hervé et al., 2004).

Feeding female rabbits with mycelium from Fusarium roseum produces infertility, possibly due to contamination with the mycotoxin zearalenon, which has effects similar to those of estrogen (Nilsson et al., 1987). The impact of a large array of endocrine-disrupting compounds released into the environment as a consequence of human activities is now being investigated for potential threatening effects on the health, welfare and productivity of farm animals (Rhind, 2005). Thus, feeds contaminated by estrogen-like compounds may be responsible for sudden “outbreaks” of infertility in rabbit farms.

5.1.2. High progesterone (P+) syndrome

As has already been stated, ovulation is a neuroendocrine reflex, typically triggered by mating or induced by exogenous GnRH administration. Therefore, functional CL should not be present in the ovary of unmated rabbits or in the post partum period. Surprisingly, however, early studies (Boiti et al., 1996) showed that up to 21% of rabbits had abnormally high plasma progesterone concentrations (P+) and CL in the post partum at the time of artificial insemination (AI). These high levels of progesterone at the time of insemination, while they did not impede GnRH-induced ovulation, were indeed responsible for anti-reproductive effects, given that most of these P+ does were not receptive and did not become pregnant. Similar findings were also reported by Theau-Clément et al. (2000), who also observed the presence of two populations of

corpora lutea (CL) in the ovaries of does with high progesterone concentrations in the post partum period. In these does, the high progesterone concentration (9.4 ng/ml) was an indicator of pseudopregnancy. More recently, a negative relation between P+ does, having plasma levels greater than 1.0 ng/ml, and fertilisation was confirmed in a large study involving 840 does at different reproductive stages in the post partum period (Theau-Clément et al., 2005). Interestingly, Rommers et al. (2005) reported that in group- or colony-based systems, the number of P+ does was greater than that found in single caged females (23.4% *vs.* 0%, P<0.001). The relatively high incidence of P+ does greatly affected the fertility rate of the colony-caged does which was 76.6% compared to 95% in controls. These findings give strength to the current idea that, at least for rabbit reproduction purposes, group-housing is intrinsically related to low fertility rates, probably because the females induce ovulation by mounting each other.

As summarised in Table 1, the results of all the experiments carried out to date confirm that the high progesterone syndrome is fairly widespread and could represent a real threat to production. In all these studies, however, the underlying causes for the P+ findings were never identified.

Table 1. Summary of the experiments that have investigated the high progesterone syndrome (P+) at the time of the AI at day 11 *post partum*.

Exp.	Parity	N. of does	% of P+	Fertility % of P+ does	Reference
1	Multiparous	208	22.8	12.4	Boiti et al., 1996
2	Primiparous	170	21[(1)]	3	Theau-Clément et al., 2000
3	Multiparous	179	5.6	0	Mollo et al., 2003
4	Exp. 1 Primiparous	170	26[(2)]	18	Theau-Clément et al., 2005
	Exp. 2 Multiparous	115	10[(2)]	58	
	Exp. 3 Null+Prim+Mult	555	17[(2)]	29	
5	Single-caged	21	0		Rommers et al., 2006
	Colony-caged	47	23.4	9	

[(1)] Does having 2 CL generations [(2)]P>1 ng/mL

Progesterone is mainly synthesised in the ovary by luteal cells of the CL and steroidogenic cells of the interstitial gland. At least three basic mechanisms could explain these P+ cases: the first refers to the formation of CL as a consequence of spontaneous ovulation, the second to the increased life span of pre-existing CL due to failure or inhibition of the luteolytic mechanism, and the third to partial luteinisation of pre-ovulatory follicles. Yet another possibility emerged recently when we found that progesterone may be secreted by the adrenals following activation of the adrenal axis with ACTH (Fig. 3) and/or lipopolysaccharide-(LPS)-dependent stimulation of the immune system involving the IL-1-CRF-ACTH cascade of events (Boiti et al., 2005b).

In this latter case, poor reproductive performance of P+ does would not be the consequence of progesterone itself on fertilisation and/or embryo quality, but rather the result of other primary problems such as infectious diseases involving Gram+ bacteria or stressful conditions.

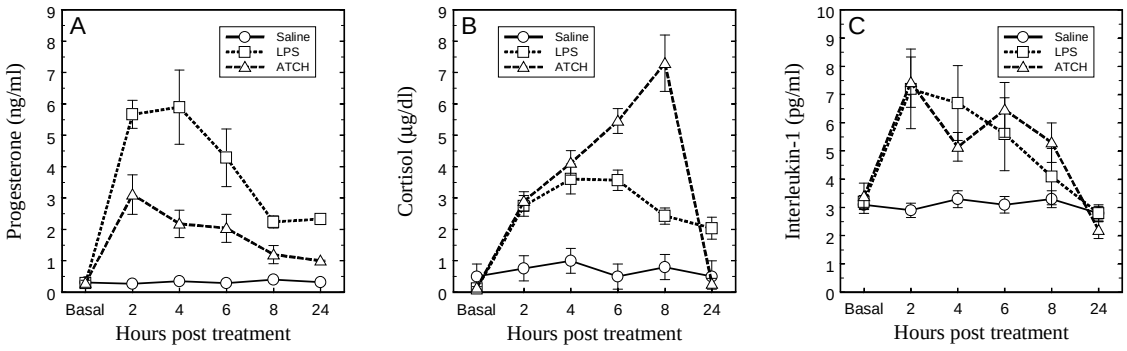

Figure 3. *Plasma progesterone (panel A), cortisol (panel B), and interleukin-1β (panel C) levels after injection of saline, LPS (100 µg/kg i.p.), or ACTH (30 µg/kg i.m) to estrous rabbits (mean ± SEM, n=7 animals/group).*

However, in our opinion, the most likely cause of the P+ syndrome is spontaneous ovulation. In fact, very high receptivity is often accompanied by a relatively high number of spontaneously ovulating does, especially in the post partum (Besenfelder, personal data). This observation is also supported, although indirectly, by plasma progesterone concentrations, when levels above 5-6 ng/ml at day-11 postpartum imply that ovulation occurred at least 4-5 days earlier, as outlined by profiles during pseudopregnancy (Fig. 4). It remains to be established, however, what factor(s) actually trigger(s) the ovulation and, in this context, a stress-related-mechanism (Xiao et al., 1996) could not be ruled out.

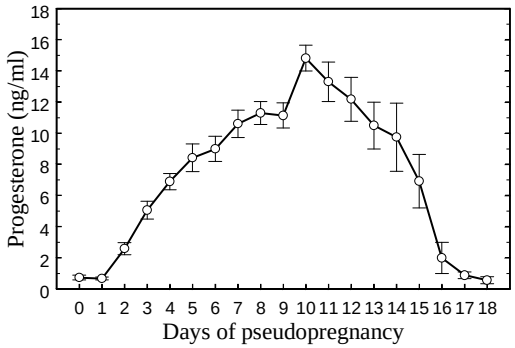

Figure 4. *Daily plasma progesterone concentrations during pseudopregnancy (Mean ± SEM). All rabbits (n=6) were injected 0.8 μg of GnRH (Day 0) to induce pseudopregnancy (Boiti, unpublished results).*

The question of whether the relatively high progesterone concentrations at the time of AI should be blamed for infertility is still being debated. Progesterone is known to inhibit gonadotropin secretion and ovulation in the rabbit (Pincus, 1940). In addition, implants of progestagens into the hypothalamic infundibular nucleus similarly blocks reflex ovulation in rabbits (Kamenatsu and Sawyer, 1965). A persistently high circulating progesterone concentration may influence the development of the endometrium.

5.2. Uterine disorders

The uterine horns and corresponding oviductal tubes play a key role in providing the appropriate internal environment for the developing embryos in the early stages of their migration into the uterine cavity, nidation, and growth until birth. Obviously, overt pathological disorders associated with either acute or chronic inflammatory diseases are incompatible with pregnancy. These disorders could be the underlying cause for those repeat breeder does not becoming pregnant after 3 or more subsequent AI treatments. At necropsy, however, these cases are easily diagnosed without resorting to auxiliary tests (bacteriological, histopathological) because of the presence of large lesions with enlarged, reddish or purple uterine horns (Fig. 5). However, much more threatening are the sub-clinic forms of the uterine component, as they are difficult to diagnose without special tools. Recently, Dal Bosco et al. (2005) reported that the recovery of spermatozoa was lower in the uterine horns of does treated with 500 μg of LPS derived from E. Coli inoculated close to the cervix 60 hours before AI. In a visual examination no significant finding was observable in LPS-treated does and it was only by histology that a mild endometritis-like inflammation was found. However, there is still much to be learnt about the relevance of immune-neuroendocrine interactions for immunoregulation, host defences and homeostasis, as well as how all these processes interfere with the reproductive sphere.

Figure 5. *In situ diagnosis of large lesions with enlarged, reddish and purple uterine horns. The photograph was taken by endoscopy 3 days after weaning and 24 hours after induction of ovulation by hCG.*

6. Conclusion

Due to the short gestation length and the large number of offspring, rabbits have gained the fame of a species with high reproductive efficiency. However, sub-fertility also afflicts rabbits, but, surprisingly, the underlying causes have not been yet closely examined. Only recently, scientific research focussed its interest on the several physiopathological aspects related to reproduction of rabbits, and novel concepts emerging from these studies may have relevance for specialists and practitioners. Unquestionably, a better understanding of the mechanisms of action involving the large array of new autocrine/paracrine putative regulators of the HPO axis function would help improve treatment of sub-fertility and adopt new management strategies better tailored to rabbit production. The study of the mechanisms involving neuroendocrine-immune interactions and pregnancy-related immunoregulation of gametes and embryos

represents a promising new frontier for the treatment of infertility and pregnancy disorders including abortion.

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1.2. Alternative methods for the synchronisation of oestrous in lactating rabbit does

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Introduction

Artificial insemination (AI) is widely used on European rabbit farms. This method provides new systems of production such as "cycled production" in which all the does in a batch are inseminated on the same day, whatever their sexual receptivity. Theau-Clément and Roustan (1992), Castellini and Lattaioli (1999) found a particularly strong antagonism between lactation and reproductive functions in non-receptive does. At the time of insemination, lactating non-receptive does showed poor performance. This antagonistic effect represents a major problem since the intensive production in general use requires does to be inseminated during the first phase of lactation (from 0 to 11 days post partum). It should be emphasised that with natural mating, the negative effect of this antagonism is hidden, since non-receptive does refuse to mate. Sexually receptive behaviour is correlated with more pre-ovulatory follicles on the rabbit ovary (Kermabon *et al.*, 1994) and consequently with higher concentration of plasma oestradiol (Rebollar *et al.*, 1992). To ensure regularly high production levels it is therefore necessary to employ reliable techniques to induce and synchronise oestrus (leading to sexually receptive behaviour) in lactating does.

Several studies have been made on hormone treatments (Maertens *et al.*, 1995b; Castellini, 1996). Pregnant Mare Serum Gonadotrophin (PMSG or eCG) is largely used in rabbitries. Nevertheless, the use of exogenous products (hormones, antibiotics, etc.) does not find favour with consumers. In the near future, European Community policy could impose restrictions on the use of hormones (gonadotropins) in relation to their residues in meat, animal welfare and the desire to preserve the "natural" image of meat. There is a similar trend concerning the use of antibiotics; in order to counteract antibiotic resistance the European Union has already banned the use of antibiotics as growth promoters in animal feeding as of January 2006 (EC 1831/2003).

For the foregoing reasons important work has been done in recent years, particularly by the International Rabbit Reproduction Group (IRRG), to find alternative methods which do not require the use of hormones to increase sexual receptivity at the time of insemination and consequently the productivity of rabbit does (Boiti, 1998). These are often called "biostimulation" methods. They are applied shortly before insemination and include a wide range of techniques. Since the year 2000, within the framework of COST 848, different approaches have been tried such as animal manipulation, a short period of dam-litter separation, a feeding program, a photoperiod and the male effect. Some of these methods were adopted after

proving successful in other zootechnical species. After a short section devoted to the physiological background and response to environmental stimuli, different alternative methods for the oestrous synchronisation of lactating rabbit does will be presented. The aim of this chapter is to analyse the different methods in the light of zootechnical results, feasibility under farming conditions, compatibility with animal welfare and physiological interpretation, as well as any promising avenues of exploration.

Physiological responses to environmental stimuli

Reproduction is regulated by a complex neuro-hormonal system in which the hypothalamus and the pituitary gland play leading roles. The secretion of GnRH (Gonadotrophin Releasing Hormone) produced at the hypothalamus level is able to stimulate both the synthesis and release of two gonadotropins: FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone) at the anterior pituitary level. These protein hormones act upon the ovaries: FSH is mainly responsible for follicular growth and LH controls the final follicular maturation and induces the ovulation of pre-ovulatory follicles.

In most species, including the rabbit, the ovarian steroid hormones (oestrogen and progesterone) seem to alternately exercise a positive and negative feedback, respectively for oestrogen and progesterone, on the secretion of GnRH, FSH and LH in the hypothalamo-pituitary complex. This whole system regulates the sexual activity of does. Moreover, complex mechanisms (described by Boiti *et al.* in chapter 1.1) interfere with the hypothalamo-pituitary-ovarian axis with the participation of endogenous opioid peptides such as endorphins, catecholamines (including DOPA and Norepinephrine), corticotrophin releasing hormone (CRH), adrenocorticotropin hormone (ACTH) and cortisol (Boiti, 2004).

It has long been recognised that the environment plays an important role in the regulation of the reproductive function and it now appears obvious that environmental stimuli must act through the nervous system and the hypothalamo-pituitary axis. Environmental stimuli, such as changing day-length or temperature and feeding, by affecting animals via stressful, auditory and/or olfactory stimuli, can positively or negatively modify reproductive performance.

Biostimulation methods

1. Animal manipulation

1.1. Change of cage

In nulliparous rabbit does, Lefèvre and Moret (1978), and Rebollar *et al.* (1995) found that a change of cage can improve fertility. In contrast, Luzi and Crimella (1998) did not confirm such an improvement on nulliparous does by transferring does (and their litter when lactating) to another cage, 2 days before insemination. However, a change of cage 48h before insemination increased fertility (+14%), compared to the control group, and resulted in 1.4 more born alive/AI in lactating pluriparous animals. Rodríguez-De Lara *et al.* (2003) found that relocation of does to another room in cages separated from their young 8-10 h before AI, resulted in more total kits born without any change in fertility.

1.2. Doe gathering

Mirabito *et al.* (1994a), by gathering 3 rabbit does immediately before insemination, did not obtain any improvement in performance, even in nulliparous does. Duperray *et al.* (1999) studied the interest of doe gathering (8 animals/cage, 15 min before AI) when dam-litter separation was applied to both experimental and control groups. This stimulation increased the frequency of red and purple vulva suggesting a positive effect on rabbit doe receptivity and significantly increased fertility (+6%). Nevertheless, the positive effect on fertility is clear on nulliparous, multiparous lactating and non-lactating does, but not on primiparous rabbit does. At birth, the size and the weight of the litter are not modified by the treatment. Productivity at birth is increased by + 0.6 born alive/insemination when doe gatherings are applied in addition to a dam-litter separation. However, this biostimulation method can only be used on healthy herds, since the direct contact between animals could represent a source of contamination. Moreover, gathering of does could lead to a risk of aggressive behaviour (injuries to rabbits).

In conclusion, the efficiency of animal manipulation in increasing sexual receptivity and rabbit productivity has not been clearly demonstrated, since the results obtained by different authors are often contradictory. Such biostimulation methods are also time-consuming and difficult to apply, on large rabbit farms, as they require sanitary control, individual identification and frequent changing of cages.

2. Dam-litter separation

It is well known that shortly after weaning (2-3 days), a high percentage of does enter oestrus (Theau-Clément and Roustan, 1992). Nevertheless, regular post-weaning insemination (with no competition between pregnancy and lactation) is likely to be cost-effective. During lactation, a short Dam-Litter Separation (DLS), has been shown to potentially induce oestrus. In sows, a daily dam-litter

separation from 6 to 12 hours at 2 to 5 weeks post partum induces oestrus in 65% of the dams, compared with only 50% in the control group (Stevenson and Davis, 1984). In rabbits, a short (24 to 48 hour) dam-litter separation has been studied, generally by closing the nest box. Moreover, in order to avoid early young mortality, farmers often use controlled nursing during the first half of pregnancy. This consists of opening the nest box each morning at a certain time for 15 to 30 minutes and closing it again until the next morning.

In order to precisely define the optimal method, different application conditions were studied (see Table 1):

- nursing system (free or controlled nursing, or a combination of both)
- separation length
- AI time in relation to the first nursing following dam-litter separation (from 2 days before AI to 2h after the beginning of the controlled nursing).

2.1. Effect of DLS on reproductive performance and productivity

The efficiency of these methods will be assessed by successively examining the sexual receptivity induction, productivity components (fertility and prolificacy, young viability and growth) and global productivity. Since rabbit farms generally use a 42-day rhythm, the studies presented in this subchapter concern 10 or 11-day lactating does. Nevertheless, some results obtained with a 35-day reproduction rhythm will also be considered.

2.1.1. Free nursing applied before and after separation

A 24h DLS, on the 3rd day before insemination.

Castellini *et al.* (1998) compared two different DLS techniques lasting 24 hours. The separations were performed on the 8th lactation day by closing the nest box or by a change of cage (which involves a DLS in addition to a modification of the does' micro-environmental conditions) with a return to free nursing the next day and AI at 11-day post partum. These two methods of mother-litter separation, probably applied for too short a time and/or too early in relation to AI, did not affect the reproductive performance of lactating does.

In the following studies, DLS was performed just before insemination and lasted from 24 to 48 hours. The reproductive performance and growth of young are presented in Table 1.

A 24h DLS, just before AI (insemination performed in the 15 min following the first suckling after DLS).

This stimulation improved the sexual receptivity and fertility of 11-day lactating does in some experiments (Pavois *et al.*, 1994; Theau-Clément and Mercier, 1999), but not in others (Alvariño *et al.*, 1998; Maertens *et al.*, 2000; Theau-Clément *et al.*, 2003). These conflicting results in the two studies of Theau-Clément and Mercier (1999, 2003) are surprising and remain unexplained. In the first study, stimulation increased the fertility of the experimental group despite the high level of fertility of the control group (94.9 vs. 82.3%, respectively), whereas in the second, the stimulation failed to improve on the poor fertility of the control group (52.1 *vs*. 57.0%, respectively). The only difference in the experimental design was the time of insemination following an unsuccessful one (first study: 2 batches, re-insemination 3 weeks after the previous one; second study: single batch).

A 24 h DLS before AI has no effect on litter size and young viability. Theau-Clément and Mercier (1999, 2003) found that the kits separated from their mother 24 h before AI were lighter at 11 and 21 days of age but the differences were no long significant at weaning (35 d).

When free suckling is applied before and after a short DLS, fertility can be improved but, at the same time, growth of young is slightly diminished. In order to evaluate more precisely the effect of DLS as a biostimulation method, two productivity indexes were calculated, with available bibliographic data (using a 42-day reproductive rhythm, free suckling before and after the stimulation and studying at least 2 series of inseminations):

- productivity at birth: the number of born alive/number of inseminations,
- productivity at weaning: the total weight of rabbits obtained/ number of inseminations.

In comparison with a control group (no stimulation), the relative differences in productivity at weaning with different DLS protocols are presented in Table 1. The positive effect of a 24-hour DLS on the global productivity was reported for only two studies in comparison with the control group: Pavois *et al.* (1994) with + 16% born alive/AI and Theau-Clément and Mercier (1999) with + 19% of weight of weaned rabbits/AI), suggesting that the stimulation was often insufficient to systematically improve the receptivity and consequently the productivity of rabbit does at weaning.

A 36-48h DLS, just before insemination.

This separation length involves the omission of at least one nursing. On 11-day lactating does, in comparison with a control group, the sexual receptivity was often improved (Pavois *et al.*, 1994: +23%, Maertens, 1998: +38%, Bonanno *et al.*, 2000: +21%, Bonanno *et al.*, 2005: +27%) as well as fertility (from 11 to 24%).

On 4-day lactating does, Alvariño *et al.* (1998) found a greater effect for biostimulation on fertility (36h: + 32%; 48h: + 34%). Generally, a 36-48h DLS

Table 1. Reproductive performance of 11-day lactating does briefly separated from their litter, in comparison with a control group (without separation).

Nursing system before and after D.L.S [(1)]	**Separation length**	AI position / 1st suckling following DLS[(1)]	*Authors*	Receptivity (%)	Fertility (%)	Born alive /litter	Weaned /litter (age in days)	Individual weaning weight	Weight of weaned rabbits /AI (%)
Free suckling	**24 h**	15 minutes after	*Pavois et al. (1994)*	+ 26%	+ 13%	NS	-	-	+ 16% (birth)
			Alvariño et al. (1998)	-	NS	NS	NS (32)	- 36 g	-
			Theau-Clément et al. (1999)	+ 8%	+ 13%	NS	NS (28)	- 34 g	+ 19%
			Maertens et al. (2000)	-	NS	-	NS (28)	NS	-
			Theau-Clément et al. (2003)	NS	NS	NS	NS (35)	NS	NS
idem	**36 h**	*idem*	*Pavois et al. (1994)*	+ 23%	+ 11%	NS	-	NS	+ 14%
			Alvariño et al. (1998)	-	+ 11%	NS	NS (32)	-73 g	-
idem	**40 h**	*idem*	*Maertens (1998)*	+ 38%	+ 11%	+ 1.1	NS (28)	- 47 g	+ 9%
idem	**48 h**	*idem*	*Alvariño et al. (1998)*	-	NS	NS	NS	- 68 g	-
			Bonanno et al. (2000)	+ 21%	+ 23%	NS	NS (35)	NS	+ 28% (70d)
			Bonanno et al. (2002)	NS	+ 24%	NS	+ 0.3 (35)	- 38 g	+ 54 %
			Bonanno et al. (2004)	NS	+ 17%	NS	+ 0.3 (35)	- 48 g	+ 35%
			Bonanno et al. (2005)	+ 27%	+ 18%	NS	NS (35)	NS	+ 25%
idem	**48 h**	A.I just before nursing	*Virag et al. (1999)*	-	+ 20%	NS	NS (30)	- 27 g	+ 20%
Controlled nursing	**48 h**	-2h, 15 min after, + 2h 15 minutes after	*Szendrö et al. (1999)*	NS	NS	NS	-	- 34 g	-
			Bonanno et al. (2000)	NS	NS	NS	NS (35)	NS	+ 7% (70d)

[(1)] DLS: Dam-Litter Separation, NS: Non Significant (P>0.05)

does not affect litter size. Only Maertens (1998) obtained a higher litter size when a 40h dam-litter separation was applied (8.2 *vs*. 7.1 born alive). Moreover, there is general agreement that neither the incidence of mastitis nor young rabbit mortality is affected by a 40-48 h DLS (Maertens, 1998; Bonanno *et al.* (1999a,b, 2004).

When weaning takes place between 28 and 32 days, a DLS longer than 24h generally impairs growth of the young, as the individual weaning weight decreases from 6% to 10% (Alvariño *et al.*, 1998; Maertens, 1998; Szendrö *et al.*, 1999). But when the same mother deprivation protocol is applied to younger rabbits (4 days old, Alvariño *et al.*, 1998), the decrease of weaning weight at 28 days of age is greater than 10%. This suggests a differential sensitivity in relation to age, but the experiment did not separate the effects of the age of the kits at the moment of mother deprivation from their weaning age.

Nevertheless, when weaning is delayed until 35 days, the decrease in the individual weaning weight can be lower than 6% (Bonanno *et al.*, 2002, 2004). Thus, most of these studies indicate that a 36-48 h mother deprivation leads to a lower weaning weight in young rabbits.

DLS can influence doe's feed intake and milk production. Maertens (1998), applying a 40h dam-litter separation, registered a decrease in feed consumption between day 8 and day 11 post partum (282 *vs*. 341 g/ day for the control group). Similarly, Bonanno *et al.* (1999b) observed a reduction of does' feed intake by 38 g DM/Kg $W^{0.75}$ on day 10 during a 48h DLS. When kits were weighed immediately after suckling, following a 48h separation, Szendrö *et al.* (1999) found a marked fall (- 13%) compared to the control group. Moreover, the day after the omission of suckling, the quantity of milk produced by the stimulated does increased by 22% on the three subsequent days. In addition, two days after the omission of suckling, the milk secreted was found to contain higher levels of dry matter (by 4.2%), fat (by 1.7%), protein (by 2.6%) and ash (by 0.5%) than previously. These values later returned to levels approaching those before separation. Nevertheless, the compensation of milk production is not large enough to counterbalance the negative effect of the separation on young growth till weaning. During fattening, Bonanno *et al.*, (1999b, 2000) observed no difference in daily gain between 48h DLS and control rabbits; therefore, DLS rabbits maintained the gap on weight and did not exhibit compensatory growth, but the weight reduction accounted for only 3% and 2% at 74 and 71 days of age, respectively. A similar result (-2% at 70 days of age) was published by Szendrõ *et al.* (1999).

A 36-48 h DLS can impair the growth of young. Under the same experimental conditions, Alvariño *et al.* (1999) found that young weight measured (in comparison with the control group) after suckling the day of insemination, decreases in relation to the separation length (-1.4%, -6.1%, -12.8%, respectively after 24, 36 and 48 h DLS).

A 48h DLS, insemination at the end of the separation, just before nursing.

Virag *et al.* (1999) confirmed the improvement of fertility when the does were inseminated at the end of a 48h DLS but just before nursing (64.7 *vs*. 44.9% for the control group) without any effect on prolificacy.

The productivity at weaning (Table 1) is systematically improved when a 36-48 hour DLS is applied (in comparison with a control group, 36h: +14%, Pavois *et al.*, 1994; 40h: +9%, Maertens, 1998; 48h: +28% Bonanno *et al.* 2000, +54% Bonanno *et al.* 2002, +35% Bonanno *et al.* 2004, +25% Bonanno *et al.*, 2005, +20% Virag *et al.*, 1999). These results are in agreement with Duperray (1995), who concluded in a field experiment that a 36 hours DLS associated with free suckling and a feed additive, increases the fertility rate (+ 8.5%) in about 70% of rabbitries without any negative impact on the doe and the litter.

2.1.2. Controlled nursing applied before and after the separation

Since controlled nursing (once a day, i.e. 24 h DLS) is largely used on rabbit farms, some authors have studied the efficiency of a 48 hours DLS when controlled nursing is applied before and/or after the insemination (Table 1).

Receptivity and fertility were not improved by a 48h separation when controlled nursing was applied from 0 to 18 days post partum (Szendrö *et al.*, 1999) or from 0 to 9 days post partum (Bonanno *et al.*, 2000). This biostimulation did not influence the litter size at birth. Szendrö *et al.* (1999) confirmed a decrease in the growth of young (- 34 g) whereas Bonanno *et al.* (2000) registered a non-significant difference at weaning. However, at the end of the fattening period (70 days), the productivity was higher (+ 7%) when the does were submitted to a 48 h DLS.

The reproductive performance of rabbit does was not improved when controlled nursing was practised before and after a 48 hour dam-litter separation. Nevertheless, a slight effect has been noted when AI was practised immediately after the first suckling following the separation (Szendrö *et al.*; 1999: +0.9 born alive/insemination) on the other hand, when AI is carried out 2h before or 2h after suckling, the separation did not have any positive effect on the reproductive performance of does. Practising AI immediately after the first nursing following the separation can therefore be recommended.

Table 2. Reproductive performance of 11-day lactating does when nursing method is changed from free to controlled nursing[(1)] (CN) 2 or 3 days before AI comparison with a control group (free access to the nest box, from kindling to weaning).

Controlled nursing length before AI	after AI	AI position / 1st suckling following the last CN[(1)]	*Authors*	Receptivity (%)	Fertility (%)	Born alive /litter	Weaned (35 days)	Individual weaning weight	Weight of weaned rabbits /AI (%)
2 days	**0** day	15 minutes after	*Matics et al. (2004b)*	NS	NS	NS	NS	NS	-
			Eiben et al. (2004b) [(2)]	-	+ 17%	NS	NS	+ 29 g	+51%
			Bonanno et al. (2004)	NS	+ 15%	NS	+ 0.5	NS	+44.%
			Bonanno et al. (2005)	+ 18%	+15%	NS	NS (30d)	NS	+ 21%
2 days	**3** days	*idem*	*Eiben et al. (2004b)* [(2)]	-	+ 27%	+ 1.6	NS	+ 34 g	+ 86%
			Eiben et al. (2004b) [(3)]	-	+ 26%	NS	NS	+ 37 g	+ 76%
2 days	**7** days	*idem*	*Eiben et al. (2004a)* [(3)]	NS	NS	NS	NS	- 67 g	+ 26%
3 days	**0** day	*idem*	*Matics et al. (2004b)*	+ 21%	NS	+ 1.2	+ 1	NS	-
			Szendrö et al. (2005c)	-	+ 9%	NS	NS	-20g	+ 5%

[(1)] During 2 or 3 days before AI, the nest box is closed from 24 hours from 9 or 10:00 h of day X to 9 or 10:00 h of day X+1, the nest box is closed again after a controlled nursing lasting maximum 15 minutes.
[(2)] The nest box is removed and not only closed
[(3)] A wire mesh is inserted during the separation
NS: Non Significant (P>0.05)

Consequently, studies applying controlled nursing before and after DLS did not lead to a systematic increase of productivity at weaning. Regular controlled nursing could limit or suppress the positive effect of a single DLS on reproductive performance. Therefore, free suckling both before and after the separation is recommended to ensure the success of DLS.

2.1.3. Alternating suckling systems

To limit the adverse impact of longer DLS on weaning weight, some authors have originally studied the influence of 2 or 3 days of controlled nursing immediately before insemination. This corresponds to a 2 x 24h or a 3 x 24h DLS respectively, allowing young rabbits to suckle when the nest box is opened (for 15 to 30 min in the morning). Controlled nursing is sometimes prolonged from 3 to 7 days after insemination (Table 2).

Apart from the experiment of Matics *et al.* (2004b), who obtained high fertility (78%) in the control group, a 2-day controlled nursing prior to AI, increased fertility (from 15 to 17%: Eiben *et al.*, 2004b; Bonanno *et al.*, 2004, 2005). When young rabbits were allowed to suckle every day, there was no negative effect on young growth, and occasionally weaning weight was even increased (Eiben *et al.*, 2004b). This could be explained by the higher frequency of daily nest visits when DLS does returned to free nursing (Matics *et al.*; 2004a). Consequently, when 2-day controlled nursing is applied until AI, productivity can be greatly increased (Eiben *et al.* 2004b: + 51%; Bonanno *et al.* 2004: + 44%, Bonanno *et al.* 2005: +21%).

Prolonging the duration of controlled nursing to 3 days after insemination increased fertility and litter size, and consequently productivity (+ 25-35% of the weight of weaned rabbits/AI, compared with a 2 x 24h DLS, Eiben *et al.* 2004b). However, longer controlled nursing after AI (7 days) has a detrimental effect on young growth, but in comparison with a control group characterised by weak fertility (33%), productivity is increased by + 26% (Eiben *et al.* 2004a).

Applying a 3-day controlled nursing before insemination, Matics *et al.* (2004b) did not succeed in increasing fertility (78 *vs*. 80%) but did increase litter size at birth and at weaning by 15 and 14% respectively. In another experiment, Szendrö *et al.* (2005c) used the same method and fertility increased by 9% (from 72 to 80%) with no significant effect on litter size. Since the individual weight at 3 weeks of age decreased (-20g), the productivity increased by 5%. Nevertheless, Eiben *et al.* (2004b, 2005ab) found that the method of separation influences productivity. A wire-mesh (which permits visual, olfactory and acoustic contact) is less efficient than a metal plate (allowing olfactory and acoustic contact but inhibits visual communication) or removing the kits (no visual, olfactory and acoustic contact).

2.2. Efficiency of DLS related to the physiological status of the does

2.2.1. Physiological status of the does

The efficiency of DLS can depend on the physiological status of the does at the time of insemination. A short DLS (Fig. 1) or a controlled nursing applied 2-3 days before AI (Fig. 2) mainly influences fertility. In all the published studies examined here, the high variability in the fertility of the control group (from 33 to 82%) is worthy of note and illustrates the limits of knowledge concerning the physiology of rabbit does.

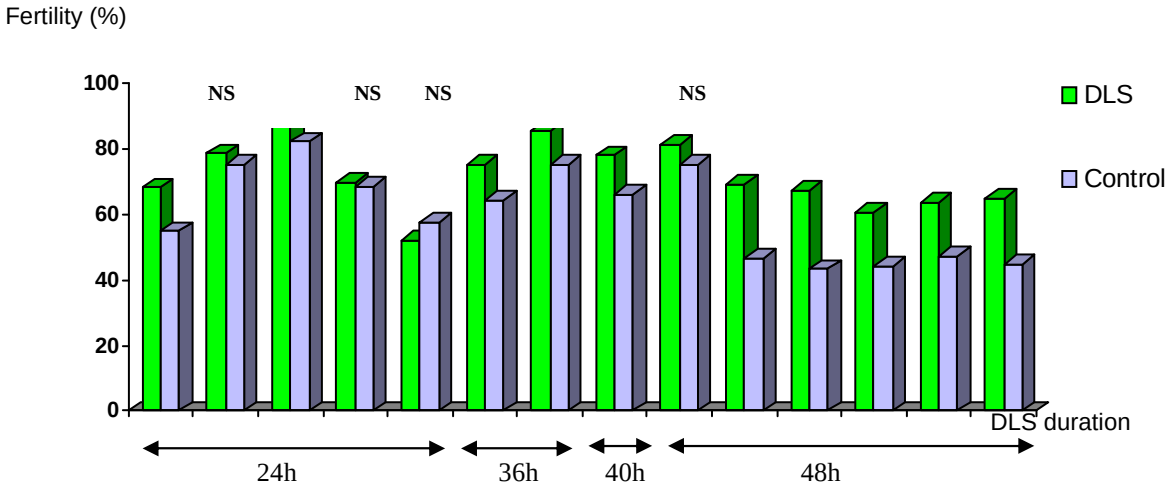

Figure 1. *Influence of a DLS (and its duration) before AI on the fertility of 11-day lactating does, when free nursing is applied before and after the separation* (*The results are presented in the same order as in Table 1). NS: not significantly different from controls*

Moreover, as evidenced by Tables 1 and 2, there is often no relationship between receptivity and fertility, which could be interpreted as a consequence of the inaccuracy of the subjective evaluation of the vulva colour and turgidity as an indicator of does' sexual receptivity.

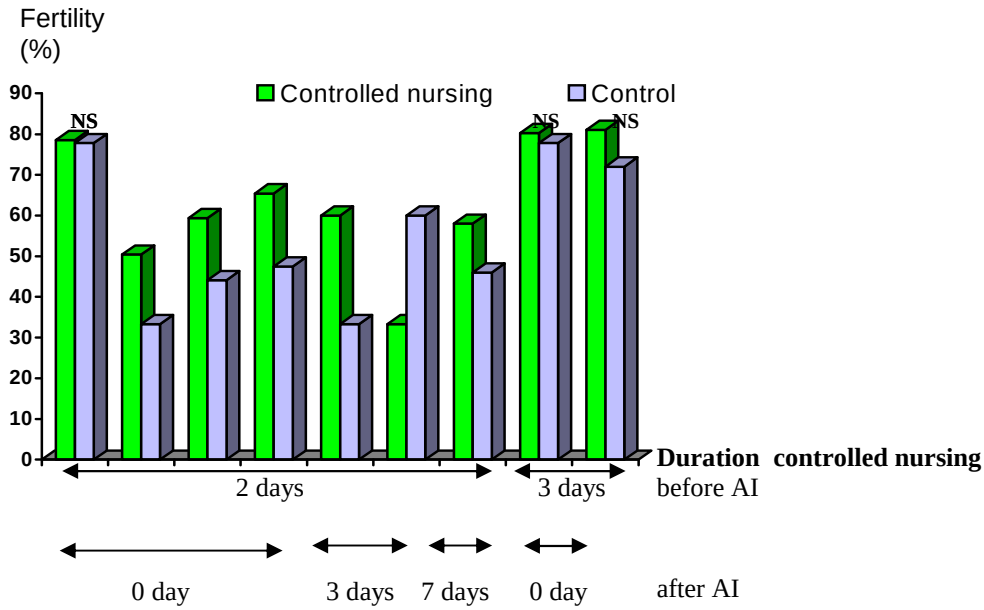

Figure 2. *Influence of controlled nursing (and its duration) before AI on the fertility of 11-day lactating does, when free nursing is applied before and after the separation (The results are presented in the same order as in Table 1). NS: not significantly different from controls*

2.2.2. Lactation stage

Stimulation applied to 3-4 day lactating does (35-day reproduction rhythm) has a greater effect on reproductive performance than with 9-10 day lactating does (42-day reproduction rhythm). Despite the marked decrease in growth of young, a 36 and 48h DLS applied to 3-4 day lactating does can improve productivity at birth by 76 and 92% respectively (Alvariño *et al.*, 1998). Theau-Clément and Roustan (1992) indicated that the antagonism between lactation and reproduction is specially marked during the first 3-5 days of nursing. The possibilities of improving production are consequently greater during this period.

Sexual receptivity: Using both free and controlled nursing, Tomas *et al.* (1996) did not find any positive effect of DLS with natural mating (i.e. with receptive does) on productivity. On the other hand, Bonanno *et al.* (1999b) demonstrated that a 48h separation with non-receptive does (lactation order<5) can improve productivity at weaning by +58%. These results suggest that dam-litter separation also improves the reproductive performance of non-receptive does.

2.2.3. Parity

Analysing the effect of a 40-48 h DLS in relation to parity, Maertens (1998) and Virag *et al.* (1999) observed that fertility is mainly improved in primiparous does (+ 30% and + 43%, respectively). Bonanno *et al.* (2000, 2002, and 2005) found that a 48h DLS improved fertility (from 19% to 35%) only on the 1-3 lactation order, whereas the treatment was ineffective on does of higher parity. In the same way, a 48h separation improved the fertility (+ 25%) of non-receptive does whose lactation number was lower than 5 (Bonanno *et al.*, 1999). It is interesting to observe that in this case the authors did not find any significant decrease in the weaning weight. However, Bonanno *et al.* (2002) found that when DLS was repeatedly applied to does that had produced more than 3 litters, fertility progressively dropped and became similar to that of the control group, and the growth reduction occurred only after 3 successive DLS treatments. This result suggests that the effect of DLS on fertility and growth of young could also depend on the number of successive treatments. Further studies will be necessary to investigate this differential age and/or parity dependent effect of DLS on fertility.

The foregoing studies indicate that many different factors can effect the efficiency of dam-litter separation: the conditions under which biostimulation is applied (the suckling system before and after stimulation, duration of separation, interval between suckling following the separation and insemination) and the physiological status of does (lactation stage, sexual receptivity at the time of insemination, parity, etc.). Moreover, additional

factors, such as genetic factors, nutrition, breeding conditions, etc. could also interact with the effect of dam-litter separation. Consequently, each of the components of global productivity, such as fertility and growth of young may be influenced in different ways.

2.3. Physiological aspects

From the physiological point of view, several hypotheses can explain the positive response of DLS on reproductive performance. When the separation varies from 24 to 48h, one or more sucklings are omitted. Ubilla *et al.* (2000, 2001) studied the pituitary and ovarian responses to a 48h dam-litter separation in nursing rabbits. They obtained decreased prolactin levels 24 h after the beginning of the separation, increased estradiol-17-β concentrations the day of the insemination (confirmed by Rebollar *et al.*, 2004, on 4-day lactating does) and a greater response to GnRH treatment. These authors suggested that the lowered prolactin concentrations, probably due to the absence of suckling periods, may have stimulated the ovarian follicular growth and steroidogenesis, thus improving the receptivity and the fertility of does subjected to DLS.

A second hypothesis maintains that the effect could be related to the release of oxytocin, necessary for milk ejection. Oxytocin also affects the uterus contractions and in this way could contribute to the transport of spermatozoa to the fertilisation site, if the insemination is performed shortly after suckling.

The permanent olfactory, visual and acoustic stimuli of kits can disturb the doe and may give her a sense of insecurity that something is abnormal and induce her to leave the nest (Baumann *et al.*, 2003, Eiben *et al.*, 2004b). Moreover, this sensation may alter nursing behaviour and produce more daily visits. Thus, a third hypothesis is that doe-litter separation could act as a positive stress and influence the hormonal balance of the does. The does that used a nest box with an open entrance appeared to suffer more stress, since they responded with a higher increase of corticosterone after an ACTH challenge than the mothers using a cat-flap entrance (Bauman *et al.*, 2005). Since a progressive reduction in DLS efficiency could be linked to the number of repeated separations on the same animal (Bonanno *et al.*, 2002), stress-related mechanisms may be involved through a progressive adaptation and/or habituation to this treatment (Boiti, 2004). However, this hypothesis has never been studied, and it must be remembered that all these phenomena could interact with each other.

2.4. Welfare aspects

It is generally accepted that wild rabbits (Oryctolagus cuniculus) have only limited contact with their young and visit them briefly (only about 3 to 4 minutes) once a day to nurse before dawn (Hudson *et al.* 1996). As a consequence 24h regular controlled nursing is similar to natural conditions (Mykytowycz, 1968; Jilge, 1995) and is regularly applied under farm conditions for 10 to 18 days post partum. Verga *et al.* (1986) observed that regular controlled nursing appears to produce less emotional young rabbits. Nevertheless, several contrary findings are to be found in the literature. Recently, Schulte and Hoy (1997) found that 57% of rabbit does have between two to five nest visit periods a day, generally between dusk and dawn. In fact, Tsujii (1988), Seitz *et al.* (1997) and Hoy and Selzer (2002) observed the highest number of nursing events during the hours of darkness. Applying a 16h dam-litter separation twice a week, Seitz (1997) observed a modification of the nursing behaviour and lower weaning weight in the separated kids.

With only one exception (Eiben *et al.*, 2005ab), none of the studies comparing regular controlled nursing *vs*. free suckling, have shown a significant negative effect of controlled nursing on weaning weight (Moret, 1975; Cordier, 1978; Verga *et al.*, 1986; Le Normand *et al.* 1994; Tomas *et al.* 1996, Bonanno *et al.*, 2000, Eiben *et al.*, 2004a).

Using a single 36-48 hour DLS with free suckling before and after AI, at least one suckling event of kits is lost. A decrease of both milk intake as well as young growth until weaning has been observed, demonstrating an alteration of animal welfare. Comparing the behaviour during a 36h DLS with periods before and after the separation, Schuh *et al.* (2005), observed an increase in nervous behaviour (gnawing and scraping at entrance and walls) and suggested that a 36-hour separation of the mother from her litter can be a limitation of the species-specific behaviour.

If the nursing method is changed from free to controlled nursing some days before insemination, all does can nurse their kits every day (10-30 min. in the morning) but the nursing time is moved from night to morning and some nervous behavioural patterns were observed (Matics *et al.*, 2004a). The number of daily nursing events was not modified (1.43 *vs*. 1.44; Szendrö *et al.*, 2005b), even if the daily distribution of nursing events are modified for some days after controlled nursing. The more frequent nervous behaviour could have a positive impact on receptivity.

An interesting and unexpected side effect of DLS has emerged recently, when it was observed that early neonatal deprivation may exert long-term lasting influences during postnatal development, reducing mortality at weaning and improving subsequent fertility (Boiti *et al.*, 2001; Brecchia *et al.*, 2001).

Comparing the effects of free and controlled nursing with sliding door separation or nest box removal on maternal behaviour between 1-15 day of

lactation, Baumann *et al.*, (2005a) observed more intensive doe activity, not only in free nursing but also when nests were closed with a sliding door in contrast to the removal of kits. Baumann *et al.*, (2005b) verified that the odour emitted by the kits enhances maternal activity. More similar to natural conditions is total separation, i.e. removing the mother or its litter (Eiben *et al.*, 2004b; Baumann *et al.*, 2005c).

Consequently, from a welfare and management point of view, and with our current knowledge, it may be suggested that controlled nursing with sliding door separation applied 2 or 3 days before insemination is the best compromise, as it allows a significant increase in the productivity of lactating does without any corresponding decrease in the growth and viability of young rabbits.

When free suckling and a 42-day reproduction rhythm are used, a single 36-hour dam-litter separation could be a real alternative to hormonal treatments for inducing oestrus synchronisation and consequently improving productivity (Maertens, 1998; Alvariño *et al.*, 1998; Bonanno *et al.* 2005). The stimulation has to be performed just before AI and insemination must be practised immediately after the first suckling following the separation. Nevertheless, even this short neonatal deprivation significantly affects the growth of the young. This is why studies and recommendations are now directed towards research of the optimal use of controlled nursing. From the knowledge at our disposal, when free suckling is applied before and after insemination, 2 days of controlled nursing by closing the nest box improves productivity (higher fertility with no adverse effects on growth of young, since young rabbits are allowed to suckle once a day for 15-30 minutes). Such a method leads to the same productivity levels as a single 48h DLS or 20 IU of PMSG (Bonanno *et al.*, 2005). The benefit of prolonging controlled nursing for some days after insemination has to be confirmed, since it can be counter-productive if too long. Nevertheless, some questions still remain concerning, in particular, the durability of the effect in relation to the number of successive treatments and the parity of the does.

3. Feeding programs

It has been shown that the body weight of ewes before mating reflects the mean nutritional status of the flock and has a clear influence on reproductive performance (ovulation rate, fertility and prolificacy; Theriez, 1984). Moreover, any increase in weight just before mating has a positive effect on reproductive performance. Conversely, a lower nutritional status before mating decreases the ovulation rate and embryo viability. Hence "flushing" is commonly practised in ovine production. This consists of increasing the doe's feeding (energy) level just before mating.

Parigi-Bini and Xiccato (1993) observed large energy losses (28%) in primiparous rabbit does during lactation due to large simultaneous requirements for lactation, body growth and pregnancy. This could partly explain the low receptivity and fertility generally observed in primiparous does. Fortun and Lebas (1994) confirmed the detrimental effects of lactation in primiparous does, and showed that this effect decreases with a reduction in the number of suckling rabbits and that a negative nutritional balance in does reduces foetal growth. Nevertheless, in a recent study, Theau-Clément and Fortun-Lamothe (2005), did not find a direct relationship between the nutritional status of lactating primiparous does at the time of insemination and productivity (number of segmented ova 24 hours/AI). Because of increasing feed intake capacity with parity numbers, the negative effect of lactation should be less pronounced in multiparous does.

In short term feeding programs, flushing can be applied just before mating or insemination. Some authors have used flushing without any previous restriction, others have first applied a feeding restriction.

3.1. Flushing without previous restriction.

Fortun-Lamothe (1998b) studied the effect of stimulation with pre-mating energy intake on reproductive performance at the subsequent mating or parturition of rabbit does during 4 successive cycles. The results only suggest that increased pre-mating energy intake can have a positive effect on the conception rate. However, inadequate pre-partum energy intake (food restriction) has a detrimental effect on sexual receptivity and litter weight.

Maertens (1998) performed a 4-day flushing period with a high-energy diet before insemination on lactating does, however, this failed to improve sexual receptivity, fertility and litter size in comparison with the control group (-1.2%, -12.2% and -0.5 born alive, respectively). The author related these results to the low palatability of the experimental diet. The daily energy intake during the flushing period was lower than in the control group (-0.15 MJ ME/day). An energetic "flushing" using propylene glycol (2% in water, for 4 days before insemination) was studied by Luzi *et al.* (2001). The treatment increased fertility (64 *vs*. 53% for the control group) but did not have any effect on litter size at birth and at weaning, nor on growth of young. Despite a higher mortality between birth and weaning (21 *vs*. 14%), the treatment increased productivity at weaning by 15%.

3.2. Flushing with previous restriction.

Gosalvez *et al.* (1995) observed a positive effect of nutritional flushing 4 days before LHRH injection (following restricted feeding for 2 weeks before the

beginning of the experiment) on the percentage of ovulating 17 week-old females. To assess the impact of acute nutritional challenges on reproductive performance, Brecchia *et al.* (2004) studied the effect of 24 and 48 hour fasting just before insemination. The nutritional challenge (biostimulation) consisted in re-feeding the does of the two "fasting" groups two hours before insemination. In comparison with a control group fed *ad libitum*, the fasting applied 1 or 2 days before insemination decreased receptivity, fertility and the number of born alive in the litter. A flushing applied just 2 hours before AI is consequently insufficient. More recently, Eiben *et al.* (unpublished data) studied the effect of a 24h fasting followed by 50h of *ad libitum* feeding prior to AI in 1-14 day controlled nursing (*vs*. untreated controlled nursing or untreated free nursing does). The fasting group produced 1.6 more total born but because of the higher pre-weaning mortality and poorer body weight at weaning, productivity failed to improve (+1%) in contrast to controlled nursing without restriction (+13%).

3.3. Physiological aspects

In intensive systems, nutritional requirements are greatly increased by simultaneous pregnancy and lactation. As has often been affirmed, reproduction is affected by the body energy balance. At a physiological level, the main factors that link metabolism and reproduction (lsuch as insulin, IGFs, glucose and the neuropeptide Y), all act on the hypothalamo-pituitary axis (affecting gonadotrophin secretions) and directly on the ovaries (by altering gametogenesis; Monget, 1997). Recently, leptin, a hormone produced by the adipose cells and an important component of the energy balance regulation, has also been found to be involved in several key aspects of mammalian reproductive functions. For rabbits, only Brecchia *et al.* (2004, 2005) have described the effects of acute food deprivation on the reproductive performance of rabbit does and its impact on the hypothalamus-pituitary-ovary axis (HPO). The consequences of fasting on the HPO axis is a decrease in the expression of oestradiol-17β receptors at the hypothalamus-pituitary level and a decrease of oestradiol-17β pulse frequency and amplitude, a lower LH peak and a lower mean plasma leptin concentration.

Flushing after a restricted feeding period could improve reproductive performance, at least in young females. If feeding programmes have been demonstrated to be able to decrease reproductive performances, conversely, no study has clearly demonstrated a feeding programme able to increase the reproductive performance of lactating does without depressing young growth and animal welfare. Nevertheless, better knowledge of the underlying physiological mechanisms could help to open up new prospects in the field of rabbit oestrus induction.

4. Lighting programs

In European latitudes, Hammond and Marshall (1925) and Boyd (1986) reported that wild rabbits (Oryctolagus cuniculus) have a well defined seasonal cycle of reproduction: most pregnancies occur between February and early August with a peak in May. This means that fertility increases with increasing day-length. Walter *et al.* (1968) showed that a 16h constant photoperiod all year round reduces the reproduction problems normally associated with decreasing day length. In the same way, Uzcategui and Johnston (1992), in a study on Rex rabbits, concluded that 14 h of continuous light appears to be superior to both 10 and 12 hours for maximising doe reproduction and spermatogenesis in bucks. On the other hand, Schüddemage *et al.* (1999) carried out a one-year experiment using an intensive reproduction rhythm and found that rabbit does housed under 8h artificial light per day produced 5% more live born pups than rabbits housed under 16h light per day. In a recent study, Theau-Clément and Mercier (2004) studied the influence of lighting programmes on rabbit doe productivity, according to two genotypes (one from a nucleus selected for meat production "0067", and another for fur production "0557") and their physiological status at insemination. For a 42-day reproduction rhythm, using insemination and cycled production, the studied lighting programmes did not greatly influence global productivity. Instead of continuous 8hL:16hD, a constant 16hL:8hD is recommended for "0067" rabbit does to increase receptivity and young rabbit growth.

Other authors have studied the influence of interrupted lighting programs. Uzcategui and Johnston (1992), in Rex rabbits, concluded that intermittent lighting schedules of 10, 12 and 14 h are equally as effective as 14 h of continuous light in promoting doe reproduction. Feed consumption appears to be inversely related to total hours of light. Arveux and Troislouches (1994), submitted does to different lighting programs (continuous:16h light/day or discontinuous: 2 periods of 8h light followed by 4h dark), and increased fertility and productivity (+13% weaned rabbits/doe/year for 8hL:4hD:8hL:4hD). Nevertheless, these results were not confirmed by Theau-Clément and Mercier (2004) and Szendrő *et al.* (2005a) with regular free nursing or with controlled suckling (Szendrő *et al.*, 2004), since the conception rate, litter size, litter weight and kit mortality were similar.

4.1. Light stimulation

By modifying the lighting program (8h light/day until 8 days before insemination followed by 16h

light/day immediately after), Theau-Clément *et al.* (1990) in comparison with a control group (constant 16 h light/day), found a significant improvement in the sexual receptivity of does (71.4 *vs*. 54.3%). However, the effect on fertility was not significant (61.4 *vs*. 48.9%). Because of lower viability and weaning weight (-5%) between birth and weaning, productivity at weaning was not improved by the experimental lighting program. Mirabito *et al.* (1994b), using a similar lighting program (except that in the week after AI there was a progressive return to 8h/ day) but with a longer reproductive rhythm (6 *vs*. 5 weeks) obtained significantly higher receptivity and fertility in lactating does of the experimental group (+10%), but litters were lighter at weaning (-6%). Maertens and Luzi (1995a) submitted rabbit does to a 16hL:8hD continuously lighting schedule or to a 10hL:14hD schedule. In order to synchronise the oestrus, the lighting period of the experimental group was suddenly increased to 16hL 5 days before insemination. No effect was observed on reproductive performance. However, in these studies, young viability or litter weight at weaning was significantly lower in the treated groups, suggesting that the lighting program can adversely affect the milk production of females and/or the feeding behaviour of young rabbits.

4.2. Physiological aspects

The physiological mechanisms by which the photoperiod acts on the reproductive functions are not well known in rabbits. Lighting programs are widely used in avian species. In sheep and goats, the photoperiodic information is transmitted via the retina to the pineal gland as nervous impulses. Knowledge of the different effects of the photoperiod on neuroendocrine pathways and reproductive activity in these species has led to the successful application of light treatments to control seasonal reproductive activity (Chemineau *et al.* 1992).

4.3. Welfare aspects

In the field of lighting programmes, few works have included a study of rabbit behaviour and welfare. Despite the lack of influence of lighting programmes on young growth, Szendrő *et al.* (2005b) found a modification in the number of daily nursing events. Under a 16h constant lighting photoperiod, most of the does nursed their kits during the dark period, but under a 8L:4D:8L:4D interrupted lighting program, most of the does nursed during the light period without any peak of nursing events.

These results illustrate the need to study photoperiodism in the rabbit more intensively. Since the preferred time of nursing activity could interact with the lighting programmes, the feed consumption and milk production of the mother have to be studied, so as not to negatively interfere with young growth and animal welfare. Nevertheless, lighting programs are easy and inexpensive to apply as only dark rooms (without windows) and a light programmer are required. They will be even more efficient if all the rabbit does are inseminated at the same time. Lighting programs are thus well adapted to cycled production.

5. The buck effect

In various physiological situations, the presence of the male influences the pattern of hormonal secretions and the behaviour of the females of many Ungulate species. In ewes, (Mauléon and Dauzier, 1965), cows (Signoret, 1980) and sows (Rowlinson and Bryant, 1974) the introduction of males into the herd reduces the duration of lactational anoestrus and advances ovulation relative to the oestrus onset (Lindsay *et al.*, 1975; Poindron *et al.*, 1980) by advancing the preovulatory surge of LH (Martin and Scaramuzzi, 1983). In seasonally anoestrous ewes of several breeds, the introduction of males (if preconditioned by a period of isolation from rams; teasing) induces and synchronises oestrous (Oldham *et al.*, 1978). Likewise, the introduction of bucks among anovulatory goats, after a period of complete segregation, induces synchronous ovulations in the following days (Chemineau 1987).

In these species, olfactory cues constitute the sensory input emitted by the male at teasing. It has been found that exposure to wool collected from certain skin regions of rams which are rich in sebaceous glands will cause ovulation in seasonally anovular ewes isolated from rams (Knight *et al.*, 1980). Thus, in all cases, chemical compounds, acting as pheromones, appear to participate in the process of hypothalamic control of pituitary gonadotrophin secretions. This neuro-endocrine response can be triggered in a very short time span: in ewes, the odour of the ram induces LH secretion after only a few minutes (Poindron and Le Neindre, 1980). Other sensory cues (sight, sound and touch) are able to trigger the hormonal secretion by themselves (Cohen-Tannoudji and Signoret, 1987), but direct and sustained interaction with the ram always induces a greater response compared with mere olfactory or visual stimulation. Therefore, the different sensory information from the male has a cumulative effect. In goats, when male stimulation is sufficient and anoestrus not too deep, the conception rate and litter size of females which were anovulatory before the introduction of males, are equivalent to those of previously cyclic females (Chemineau 1987).

Accordingly, the "buck effect" has been used as an efficient method of controlling reproduction in these breeding species and appears to be a "biological" alternative to hormonal methods of stimulation, at least at certain periods of the year. So

far, we do not know whether similar mechanisms can be generalised to other taxa, such as the rabbit. Since the work of Mykytowycz's group, the paramount importance of olfactory exchanges between male and female rabbits has been clearly established. Animals of both sexes mark and overmark objects from the environment with secretions from their submandibular glands. In females, the rate of this chin marking activity is positively correlated with the state of oestrus, and it has been hypothesised that they then emit specific signals that attract males and externalise information regarding their sexual stage (Vodermayer, 1989; Hudson and Distel, 1990; McNitt, 1992). The pheromones secreted by the sebaceous glands in males could induce sexual maturity in young female rabbits (Frank, 1966). Moreover, in nulliparous rabbit does, the presence of males could contribute to an increased acceptance to mating (Lefèvre *et al.*, 1976) and an increased fertility rate (Berepudo *et al.*, 1993). Nevertheless, in recent studies, male-female proximity for a period of 4 or 48 hours (Bonanno *et al.*, 2003), 3 or 4 days (Kustos *et al.*, 2000; Eiben *et al.*, 2001) did not significantly improve the sexual receptivity and reproductive performance of lactating does. Except for the largest litter size at birth found in the cages adjacent to bucks, the distance between bucks and females did not influence sexual receptivity or fertility (Eiben *et al.*, 2001). Moreover, such stimulation is difficult to put into practice in large rabbit farms.

6. Other methods

In hot climates and also in mid Europe during summer, the high temperature has certain negative effects on productive and reproductive performance. Angora rabbits are particularly subject to heat stress because of their long hair. According to the results of Brockhausen *et al.* (1979) reproductive performance was improved when Angora rabbit does were sheared. This was confirmed by Eiben *et al.* (1997) who reported a 3% longer lifespan and 43% higher productivity in German Angora rabbits with biweekly shearing so as to maintain a coat length of 2.0-2.5 cm than in Angora does traditionally sheared one week before AI. In the study of Finzi *et al.*, (1992), the feed intake of sheared normal hair bucks was increased significantly, though neither the quantity nor the quality of semen was affected.

Szendrő *et al.*, (2004b) studied the effect of shearing does before insemination. During the summer, primi- and multiparous does were sheared on back and sides to a hair length of 2.5 mm, 2 days before insemination. In comparison with a control group, fertility and prolificacy were not improved but a slightly higher lactation curve was observed after shearing. The daily milk production between the 8^{th} and 28^{th} days was 6% higher as a consequence of shearing (lower heat stress). The litter weight at 21, 28 and 35 days was higher in the sheared group by 6, 4 and 9%, respectively. It is concluded that in summer (high temperatures), hair shearing can reduce the adverse effect of heat stress. However, shearing two days before insemination did not have any biostimulative effect on reproductive performance.

Conclusion

The aim of these studies was to examine non-hormonal methods of inducing rabbit does sexual receptivity at the moment of insemination and consequently to improve and homogenize reproductive performances avoiding, as far as possible, negative effects on young growth and animal welfare. The efficiency of animal manipulation such as a change of cage, or doe gathering, before insemination, has not been clearly demonstrated. These methods also have the disadvantages of being time consuming and are difficult to apply on large rabbit farms. A short dam-litter separation could be a real alternative to hormonal treatments (oestrus synchronisation), if the stimulation is applied just before insemination and if free nursing is applied before and after insemination. Nevertheless, controlled nursing applied 2 or 3 days before insemination (by closing the nest box or inserting a metal plate) is now preferred, since it is as efficient as DLS and more respectful to young growth and animal welfare. Nevertheless, recent studies show that besides parity, its success depends on the duration and timing. Further studies will therefore be necessary. Both nutritional flushing following a restricted feeding period and lighting programmes are interesting avenues of exploration and are open to further investigation. Unlike other zootechnical species, the first results of the effects of rabbit bucks on oestrus induction are disappointing.

Although some of these methods succeed in increasing fertility, they can reduce growth of young (lighting programs, dam-litter separation, etc.). Consequently, particular attention has to be paid to the global productivity of stimulated does, their longevity and to the duration of these positive effects. The methods have to be easy to apply, inexpensive, consistent with animal welfare and adapted to cycled production.

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1.3. New perspectives in rearing systems for rabbit does

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Introduction

In modern rabbit production, the limited reproductive lifespan of rabbit does is seen as a welfare (Blokhuis, 1995) as well as an economic problem. This is mainly attributed to the high culling rate of young does, caused by early death, disease and reproductive problems (Fortun-Lamothe and Bolet, 1995; Xiccato, 1996; Rommers, unpublished data).

During the first lactation, does lose a substantial part of their initial fat (-40%) and energy reserves (-25% to -30%) (Xiccato, 1996). Concurrent pregnancy and lactation also induce losses in nitrogen and mineral levels (Xiccato, 1996). Feed intake capacity is reported to be the main limiting factor in the negative energy balance (Xiccato *et al.*, 1995, 1996, 2004). In addition, the nutritional deficit is considered to be responsible for the decreased reproductive efficiency of young does as it presumably affects the animals' health and could shorten the lifespan of young does.

Research has focussed on improving the energy balance and performance of young does by increasing the digestible energy concentration of the diet (Maertens and De Groote, 1988; Fortun and Lebas, 1994; Pascual *et al.*, 2000) or by adopting a more appropriate reproductive rhythm (Cervera *et al.*, 1993; Parigi-Bini *et al.*, 1996; Castellini *et al.*, 2003). Up to now, however, this type of approach has only been marginally successful in compensating the energy deficit during the first lactation.

Another approach to overcoming the problems of a negative energy balance is to focus on the rearing conditions of young does. The does which are "well developed" and have an improved feed intake and/or efficient energy utilization at the start of their reproductive career could be better adapted to overcoming the negative energy balance during the first lactation. This could take the form of either an improved reproductive performance or a lower culling rate.

The objective of this chapter is therefore to take an overview of rearing methods and focus on their impact on body weight development, feed intake, productivity, and culling rate of young does during the reproductive period. The following sections will describe rearing conditions, the factors affecting body growth and development and the effects of rearing strategies on body development and feed intake. The consequences for productivity, feed intake, body weight development, and culling in subsequent reproduction will be also discussed. Finally, the practical implications will be dealt with.

1. Classic rearing conditions

In current rearing, does are often fed *ad libitum* from weaning to the first insemination, which is applied when 75% to 80% of mature body weight is reached (Lebas *et al.*, 1997), usually at around 16 weeks of age. At first insemination, body weight is around 3.5 kg. At this time most of the bone and muscle tissue has already been formed, and protein and ash content levels off at approximately 20% and 3%, respectively (De Blas *et al.*, 1977). The reproductive organs show a rapidly increasing development around 10 weeks of age (Ouhayoun, 1984) and they are considered to be sufficiently developed at first insemination. In contrast, feed intake capacity is still not fully developed, but increases during subsequent lactations until it reaches a plateau level after the fourth lactation (Castellini and Battaglini, 1991; Xiccato *et al.*, 2004).

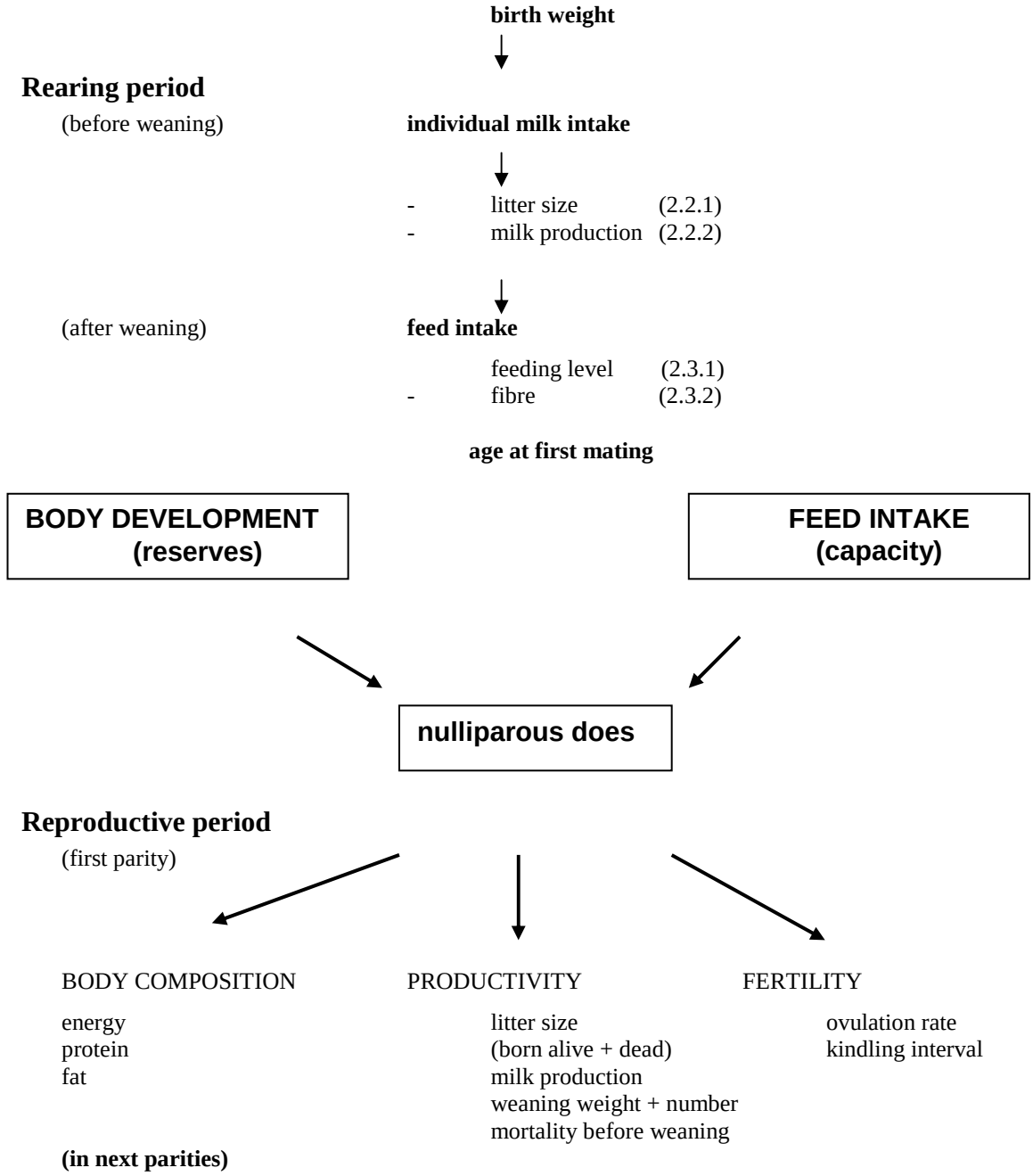

Figure 1. *Influence of management factors during different phases of rearing on body development and feed intake in subsequent reproduction.*

2. Factors affecting body growth, development and feed intake

The rearing period can be divided into the following stages:

- The period from birth to weaning (28-35 days);
- The period after weaning until first insemination (from 28-35 days until approximately 14-16 weeks of age).In the pre-weaning stage, maternal effects play an important role in the survival and development of offspring. The suckling animals are highly dependent on their mothers for nutrient intake and maternal care; under commercial conditions, kits are weaned at 28 to 35 days. A normal rearing period (from birth until first insemination at around 15 weeks of age) consists of approximately 30% suckling and 70% post-weaning period.

During the different phases of rearing, several management factors influence body development and feed intake in the subsequent reproduction period (Figure 1).

2.1. Birth weight

Weight and body composition at birth influence the development and survival of the kits during the pre-weaning period and, therefore, contribute to the development of the rabbit later in life. Heavier weights at birth result in heavier weights at 21 days and 12 weeks of age (Szendrö *et al.*, 1996; Vasquez *et al.*, 1997; Ferguson *et al.*, 1997). Therefore, birth weight seems to be an important factor in explaining at least part of the differences in growth performance.

Birth weight is influenced by several factors during the gestation period as described by Rommers *et al.* (1999). Optimal growth and development of the foetuses can be achieved when the doe is multiparous, kept under a semi-intensive reproductive rhythm (42 days), and has no restrictions in feed/energy intake.

2.2. The pre-weaning period

The period before weaning is characterized by the high growth rate of bone, heart and lung, intestines, and caecum (Cantier *et al.*, 1969; Deltoro and Lopez, 1985). Milk is the major source of nutrition. The milk production of the doe is affected by factors such as re-mating interval, parity, nutrition, and litter size (see review by Rommers *et al.*, 1999). The individual milk intake contributes to growth and development, as well as to the survival of the kits before weaning.

The number of suckling kits affects the individual milk intake of the kits, thus influencing body growth (Lebas, 1969). In smaller litters (<9-10 kits) there are more teats available than kits. Since rabbit kits switch between teats, the kits in smaller litters are able to try more teats or spend more time in switching teats in search of the most productive one (Hudson *et al.*, 1996) and in this way consume more milk.

Milk production is affected by a great number of factors: breed (McNitt and Lukefahr, 1990) or strain (Vicente and Garcia-Ximénez, 1992), and individual doe differences (McNitt and Lukefahr, 1990). Other important factors are parity, the physiological state of the doe as influenced by the re-mating interval (concurrently pregnant or not), the chemical composition of the diet, and the feed intake level of the doe (Maertens, 1992; Xiccato *et al.*, 2004). The impact of these factors on milk production has been quantified in Rommers *et al.* (1999).

2.3. After weaning

The period after weaning is characterized by a rapid development of the caecum until 5 to 6 weeks of age as a consequence of the transition from milk to solid food. Muscle (protein) tissue shows a high development rate until 10 - 12 weeks of age (Cantier *et al.*, 1969; Deltoro and Lopez, 1985). Sexual development starts around 10 to 12 weeks of age.

After weaning, body growth and development are influenced by several factors such as environmental conditions (temperature, housing density) and nutrition (feed intake level and composition of the food). Feeding level and dietary fibre level of the diet are the most important factors in stimulating feed intake and body development.

2.3.1 Feeding level.

In general, rabbits are fed *ad libitum* until slaughter weight (approximately 2.5 kg live weight) is reached. Studies on the effect of feed restriction in the period before slaughter weight is reached were carried out in order to find methods of reducing the high losses in weaning rabbits caused by diarrhoea (Maertens and Peeters, 1988) and to improve performance during fattening (Scholaut and Lange, 1990).

Only limited information is available on the effect of feed restriction and re-alimentation on the development of organs and tissues (Ferreira and Carregal, 1996; Ledin, 1984; Perrier and Ouhayoun, 1996). In all these experiments, body growth decreased during feed restrictions, mainly in the form of liver weight. Restricting intake at an early age (5-8 weeks) seemed to delay skeletal development, whereas at a later age (8 to 11 weeks) the effect was to hinder the formation of fat deposits (Perrier and Ouhayoun, 1996).

During feed restriction, feed digestibility increased and according to Ledin (1984) this effect remained when restriction was ended. When the restriction was followed by a less controlled or *ad libitum* feeding level, feed intake and body growth increased and compensatory growth took place (Gidenne *et al.*, 2003). Ledin (1984) suggested that if restriction is followed by a higher (but still restricted) feeding level, priority is given to the development of the internal organs, especially the liver. Only in the case of an excess of nutrients during the first part of the re-alimentation period will compensatory growth of other soft tissue take place. Ledin (1984) stated that during re-alimentation in her experiments the animals were trying to correct for the deviation from normal body composition caused by the restrictions, and that this would have occurred if the experiments had been prolonged.

2.3.2 Dietary fibre level

Dietary fibre plays an important role in the diet of the rabbit because of its influence on caecal microbial activity. An inadequate nutrient supply (especially fibre) can cause caeco-colic digestive disturbances, resulting in diarrhoea and mortality (Gidenne, 2003). However, the dietary fibre level also affects the digestibility of the other nutrients in the diet and can also reduce growth rate and influence chemical body composition (especially of fat content) in the growing period (Parigi-Bini *et al.*,

1994). Growth rate is not influenced if the composition of the diet is adjusted to supply an adequate amount of energy, protein, and other essential nutrients (Ortiz *et al.*, 1989; Parigi-Bini *et al.*, 1994).

Dietary fibre can be used during the rearing period to stimulate stomach development (De Blas *et al.*, 1986; Parigi-Bini *et al.*, 1994) and, therefore, to increase feed intake capacity in the reproduction period. In the growing period, increased dietary fibre levels resulted in a higher passage rate of digesta through the digestive tract (De Blas *et al.*, 1986) and increased feed intake because of the rabbit's ability to compensate for the digestible energy intake (Maertens, 1992; Parigi-Bini *et al.*, 1994). In several studies, increased empty stomach weights were found at increased fibre levels (De Blas *et al.*, 1986; Parigi-Bini *et al.*, 1994), which could be explained by an adaptation to a greater weight of feed and/or to the greater weight of soft faeces in the stomach of rabbits fed fibrous diets (De Blas *et al.*, 1986).

In conclusion, it can be stated that the dietary feed/energy level after weaning is an important factor in regulating body growth and development. Considering the body development of the rabbit, it is logical to start feed restrictions at an early age (around 5-6 weeks of age). Feed restriction before six weeks of age seems inadvisable since it will delay skeletal development (Perrier and Ouhayoun, 1996) and negatively affect caecal traits (Maertens and Peeters, 1988). Between six and 10 weeks of age, feed restrictions will delay muscle development. By increasing feed intake from 10 weeks of age onwards, compensatory growth will take place, mainly in terms of muscle development, with increased feed efficiency. By prolonging the rearing period, it should be possible to obtain a mature animal with low fat content, which might be better adapted to meeting the high demands made on it during the subsequent reproduction period.

3. Rearing strategies and their effect on body development and feed intake

Based on the information in the previous paragraph, it can be stated that does which are "well developed" at the end of rearing should be more capable of coping with the reproductive problems during first lactation. In this context, "well developed" refers to several aspects, such as:

a. Enhanced skeleton growth (to obtain more volume for feed intake and/or foetuses);
b. Higher degree of maturity, by delaying the first insemination in order to obtain a heavier animal with more body reserves in terms of protein and fat.

However, postponing the first insemination to a later stage will enable the animals to form excessive fat deposits, which could cause problems. In ruminants, it has been shown that excessive fat deposits cause health problems due to fatty liver in lactation (Rukkwamsuk *et al.*, 1999). In rabbits, increased kit mortality at kindling has been reported in does with a higher body fat content at parturition (Partridge *et al.*, 1986). Thus, to prevent excessive fattening, a form of feed restriction during rearing could be applied. Feed restriction during rearing could also be a useful tool to improve feed intake during reproduction. These mechanisms could be used to increase energy availability during the first gestation and early lactation.

c. Improved feed intake capacity, by increasing dietary fibre level in the diet.

Several authors have studied rearing strategies that focussed on body development during rearing by changing the level of feed intake during different phases of development, with or without postponing the age of first insemination. Studies on the effect of dietary fibre level during rearing are limited. In this section, the effect of rearing strategies in the period before as well as after weaning on body development and feed intake is discussed.

3.1. Milk intake before weaning

The individual milk intake of the kits is affected by number of kits within a litter (Fortun-Lamothe and Gidenne, 2000). Stimulating milk intake may stimulate skeleton growth and this may result in larger /heavier does. Babile *et al.* (1982) raised kits in litters of 5 or 11. They observed that kits reared in litters of five were significantly heavier at 120 days of age than kits raised in litters of 11 (3442 *vs.* 3097 g, respectively). Rommers *et al.* (2001a) performed an experiment in which kits were raised in litters of 6, 9 or 12 kits. Litter size affected body growth and body composition at weaning and at first insemination; kits of litters of 12 had the lowest weight and their empty bodies contained less fat at the end of the rearing period. Compensatory growth took place and after the first lactation no differences in body weight and body composition were found among litter sizes. Szendrö *et al.* (2002) found that when rabbit kits were nursed by 2 does they were significantly heavier at weaning than single-nursed kits, and that the weight difference remained until slaughter weight.

It was supposed that milk intake before weaning would enhance skeleton growth, but this was not supported by the results given above. Does raised in different litter sizes had similar ash content (Rommers *et al.*, 2001a). The reason for this is not clear, but there are several possible explanations. One could be that milk intake was not sufficiently reduced in does raised in litters of 12 kits to have any effect. Skeletal growth might have a high priority at this age, so nutrients will first of all be used for this purpose, and/or does might have been able to make up for the loss in ash content after

weaning, since feed was given *ad libitum* from weaning onwards.

3.2. Feed intake from weaning until first insemination (AI) and age of first AI

The period after weaning is characterized by the rapid development of the caecum until 5 to 6 weeks of age as a consequence of the transition from milk to solid food. For this reason it is not advisable to restrict feeding in this phase.

Feed restriction from 6 weeks of age onwards was applied by Hartmann and Petersen (1995 and 1997) at the rate of 80% of *ad libitum* fed animals. Restrictions during the rearing period negatively influenced body weight gain and resulted in lower body weight and delayed the first mating. However, after the first parturition, the restrictively reared does had reached the same body weight as the non-restrictively reared group. At the second and third parturition, they were even heavier than the non-restricted does.

Several authors (Coudert and Lebas, 1985; Van den Broeck and Lampo, 1977 and 1979; Maertens, 1984) used restricted feeding in the late rearing period (from 10-12 to 16-18 weeks of age) to reduce feed costs and prevent obesity. This mainly postponed the age of the first fertile mating. However, the negative effect was somewhat reduced when does were flushed for 5 to 7 days before the first mating.

Rommers *et al.* (2001b and 2004b) studied the effect of a feeding strategy that was better adapted to body development. Feed intake was restricted from 5 to 6 weeks of age onwards, to hinder protein and fat deposits. From 10 weeks of age onwards, feed intake was gradually increased to stimulate sexual development, which starts around 10 to 12 weeks of age. By increasing feed intake from this stage onwards, the does were able to compensate for the loss in protein building. Young does were thus prevented from developing excessive fat deposits at the first insemination, whereas sexual development was stimulated. The restrictive feeding regime (R) as described above was compared to *ad libitum* feeding (AL) and the effects on body development, feed intake capacity and subsequent reproductive performance were studied in young does that were inseminated at 14.5 or 17.5 weeks of age.

The results of the different experiments (Rommers *et al.*, 2001a, 2001b, 2004b) showed that body weight and body composition at first insemination could be manipulated by rearing strategies. Body weight at the first insemination was closely related to feeding level and age at first insemination. Depending on the rearing strategy applied, body weight at the first insemination varied between 3.2 kg and 4.2 kg (for restrictively fed does inseminated at 14.5 wk of age (R-14.5) and *ad libitum* fed does inseminated at 17.5 wk of age (AL-17.5), respectively).

In the experiments in which body composition was determined at the end of rearing (Rommers *et al.*, 2001a and 2001b), the main effect was observed to on body fat content, which varied between 14% and 24% of empty body weight (for R-14.5 and AL-17.5 does, respectively). Body protein and ash content were hardly influenced and ranged around 20%, and 3% of empty body weight, respectively, independent of rearing strategy. This is in agreement with the findings of Ledin (1984), who stated that when feed intake is restricted priority is given to organ development. In the studies of Rommers *et al.* (2001a and 2001b), protein and ash contents at the first insemination were comparable to levels reported by De Blas *et al.* (1977) for five month-old does.

3.3. Dietary fibre level after weaning

The dietary fibre level can be used during the rearing period to stimulate stomach development (de Blas *et al.*, 1986; Parigi-Bini *et al.*, 1994) and therefore increase feed intake capacity in the reproduction period. Nizza *et al.* (1997) and Xiccato *et al.* (1999) found a positive effect of increased fibre level in the diet during the rearing period on feed consumption during reproduction. Xiccato *et al.* (1999) also reported a tendency for increased feed intake in does raised on the fibrous diet during successive lactations, which resulted in a higher DE intake. Xiccato *et al.* (1999) found that does raised on a high fibre diet ate 10 kcal/d/kg $BW^{0.75}$ more and lost less body fat and body energy during their first lactation. Pascual *et al.* (2002) and Quevedo *et al.* (2005) showed that a high fibre diet during the rearing period caused growth retardation and a delay of the start of reproductive development. However, during pregnancy, the does showed a higher DE intake and corrected for the growth retardation.

4. Impact of rearing strategies on production

The main objectives of applying rearing strategies are to improve the reproductive performance of young does and to prolong their reproductive lifespan. Improved reproductive performance implies improving several reproductive factors, such as kindling rate, litter size, kit survival at kindling and during lactation, and milk production. To prolong the lifespan of does, a low culling rate during reproduction should be achieved. This section will focus on the consequences of rearing strategies on production.

4.1. Kindling rate

The size of the litter in which does are raised before weaning does not seem to have an effect on their later kindling rates (Babile *et al.*, 1982 and Rommers *et al.*, 2001a). Rearing strategy after weaning influenced kindling rate only when does were restricted in feed and inseminated at an early age (14.5 weeks; Rommers *et al.*, 2001b). A 5-day flushing period before insemination was ineffective in inducing receptivity in these does, which had underdeveloped uterine horns. According to the number of animals in which *corpora lutea* were observed on the ovaries, only 50% of the does had reached puberty. This indicated that, with the restricted rearing strategy at 14.5 wk of age, animals were too immature to start reproduction (Rommers *et al.*, 2001b). When the rearing period was prolonged by three weeks, restrictively fed does reached similar kindling rates as those fed *ad libitum* during rearing. Dietary fibre level during rearing did not affect kindling rate.

4.2. Litter size

Rearing strategy affects litter size. Heavier does, raised in litters of six or nine kits, produced more kits (+ 1.1 and + 2.2, respectively) in the first parity than small does raised in litters of 12 kits (Rommers *et al.*, 2001a). Also Babile *et al.* (1982), who raised kits in litters of 5 or 11 kits, reported a tendency for an improved total litter size and number of kits born alive for does raised in small (5 kits) litters.

Rearing strategy after weaning also affects litter size. Hartmann and Petersen (1995 and 1997), who restricted feed from six weeks of age onwards, reported that feed restriction during rearing had a positive effect on reproductive performance from the second litter onwards. The number of kits born in the second litter increased by 1.4 and the litter weight at 21 days of lactation increased in the second (+0.8 %) and third (+ 6.4 %) litters. Coudert and Lebas (1985), and Maertens (1984) reported that feed restriction during the last weeks of the rearing period reduced the number of kits born alive in the first litter.

The dietary fibre level during rearing did not affect litter size (Nizza *et al.*, 1997; Xiccato *et al.*, 1999).

4.2.1 Effect of body weight at first insemination

The experiments of Rommers *et al.* (2002) revealed that a positive relation exists between body weight at 14.5 weeks of age and litter size in the first parity for does fed *ad libitum* and inseminated at 14.5 weeks. Litter size improved from 6.4 to 8.9 kits for does weighing less than 3.5 kg compared to does weighing more than 4 kg at first insemination. Restrictively fed does inseminated at 17.5 weeks of age produced more live-born kits (+ 1.4) and weaned more kits (+ 0.6) in the first parity than does fed *ad libitum* and inseminated at 14.5 or 17.5 weeks of age (Rommers *et al.*, 2004b). Therefore, the relationship

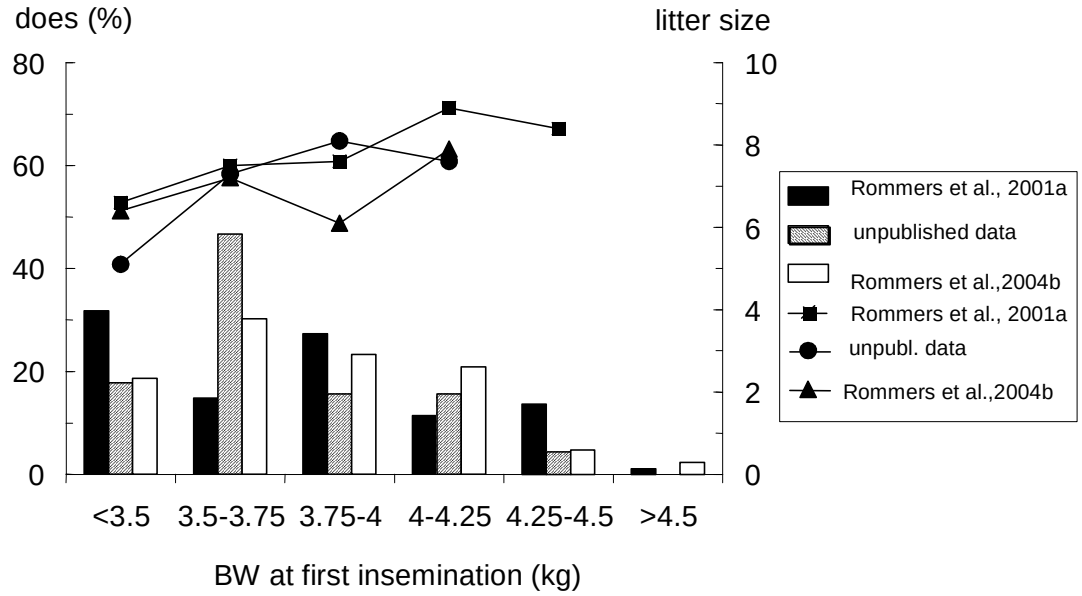

Figure 2. *Relationship between body weight at first insemination at 14.5 weeks of age and litter size in the first parity for does fed ad libitum during rearing based on three experiments. The distribution of does in different body weight classes is presented in bars. Average litter size is represented by lines.*

between body weight at first insemination and litter size in the first parity was studied in more detail. For this analysis, all available data sets were used (Rommers *et al.* 2001a, 2001b, 2002, 2004b) and the relationship for the following three rearing strategies was studied: *ad libitum* feeding and first insemination at 14.5 weeks of age (AL-14.5), or 17.5 weeks of age (AL-17.5), and restrictive feeding and first insemination at 17.5 weeks of age (R-17.5). The relationships between body weight at first insemination, and litter size in the first parity was studied by dividing body weight at first insemination into 6 body weight-classes: 1) < 3.5 kg, 2) 3.5 – 3.75 kg, 3) 3.75 – 4 kg, 4) 4 – 4.25 kg, 5) 4.25 – 4.5 kg, 6) > 4.5 kg. The outcome is presented in Figure 2 for AL-14.5 does, in Figure 3 for AL-17.5 does, and in Figure 4 for R-17.5 does.

As shown in Figure 2, litter size increases when does are heavier at 14.5 weeks of age. However, litter size seems to level off: between eight and nine kits at 4 to 4.25 kg body weight for data by Rommers *et al.* (2001a) and at 3.75 to 4 kg body weight for unpublished results. These results indicate that, at 14.5 weeks of age, does need a certain body weight (in our study around 4 kg) at first insemination to improve litter size in the first parity. This is supported by the fact that, does fed restrictively during rearing and inseminated at 14.5 weeks of age were small (3.2 kg) and showed poor fertility and embryo recovery rate (Rommers *et al.*, 2001b). It seems that body development of small does is not optimal at 14.5 weeks of age and results in a lower maturity and decreased litter size in the first parity. The fact that, under *ad libitum* feeding conditions, over 70% of the does did not reach a body weight of 4 kg at 14.5 weeks of age (see Figure 2) may explain the reduced litter size. The improved litter size of does raised in litters of six (LS6) and nine kits (LS9) in the pre-weaning period (Rommers *et al.*, 2001a) can be explained by the fact that more does (60 and 38.9%, for LS6 and LS9, respectively) weighed around 4 kg at 14.5 weeks of age, whereas only 22.9% of does raised in litters of 12 kits did so. The body development of does raised in litters of 12 kits does not seem optimal at 14.5 weeks of age. In Rommers *et al.* (2002), small (< 3.5 kg), medium (3.5 to 4 kg) and heavy (> 4 kg) does were compared for subsequent performance. The litter size of the small does was lower than that of the medium and heavy does (-1.3 and – 2.5 kits, respectively). Small does had a 25% lower growth rate during rearing. From these findings it can be concluded that does need a certain body weight (around 4 kg) to optimize litter size and, under current rearing conditions, the first insemination should be delayed until a later stage.

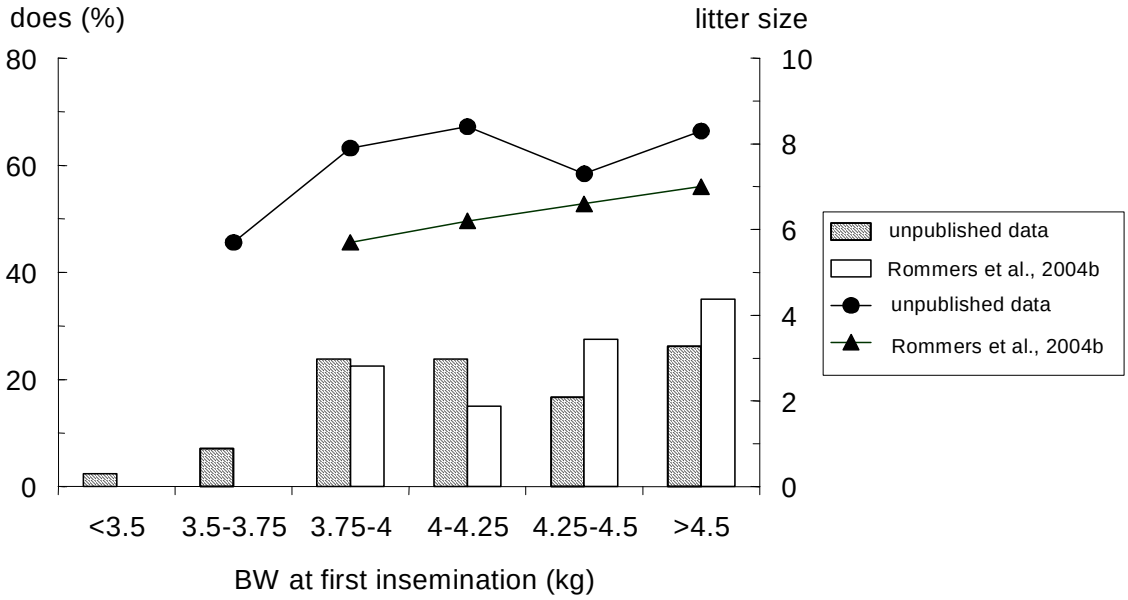

Figure 3. *Relationship between body weight at first insemination at 17.5 weeks of age and litter size in the first parity for does fed ad libitum during rearing based on three experiments. The distribution of does in different body weight classes is presented in bars. Average litter size is represented by lines.*

4.2.2 Effect of age at first insemination

To increase body weight at first insemination under *ad libitum* feeding conditions, the rearing period was prolonged until 17.5 weeks of age. The relationship between body weight at first insemination at 17.5 weeks of age and litter size is presented in Figure 3, where it can be seen that litter size in the first parity is not closely related to body weight at first insemination (at 17.5 weeks of age).

No additional increase is achieved in litter size when does get heavier than approximately 4 – 4.25 kg for does in Rommers *et al.* (2004b), and around 4.25 – 4.5 kg for does (Rommers, unpublished results). Litter size seems to level off at approximately 4 kg body weight, which is in accordance with does inseminated at 14.5 weeks of age. First insemination at an older age (17.5 weeks) improved receptivity and embryo recovery (Rommers *et al.*, 2001b), but did not result in increased litter size (Rommers *et al.*, 2004b) as compared to does inseminated at 14.5 weeks of age.

When the first insemination was postponed to 17.5 weeks of age under *ad libitum* feeding conditions, more than 75% of the does reached a body weight of at least 4 kg. However, a heavy body weight at this age gives does with a high fat content. Under this rearing strategy, does were heavier at first insemination, but this was mainly caused by deposition of fatty tissue (Rommers et al, 2001b). Based on these results, it can be concluded that the number of does that reach a body weight of at least 4 kg is increased if first insemination is applied at 17.5 weeks of age. However, some of these does will have a high fat content, which can have negative effects on gestation and lactation, as will be discussed later in this chapter.

4.2.3 Interaction between body weight and age at first insemination

To prevent overfattening at first insemination, feed intake was restricted during rearing. This period was prolonged by three weeks to enable does to restore protein and part of the fat development. At the end of rearing, R-17.5 does had similar body weight, ash, protein, and fat content as AL-14.5. Fat content was lower than AL-17.5 does (Rommers *et al.*, 2001b). Puberty characteristics of R-17.5 were similar to those of AL-14.5. Average litter size and percentage of does in the body weight classes were calculated. The outcome is presented in Figure 4.

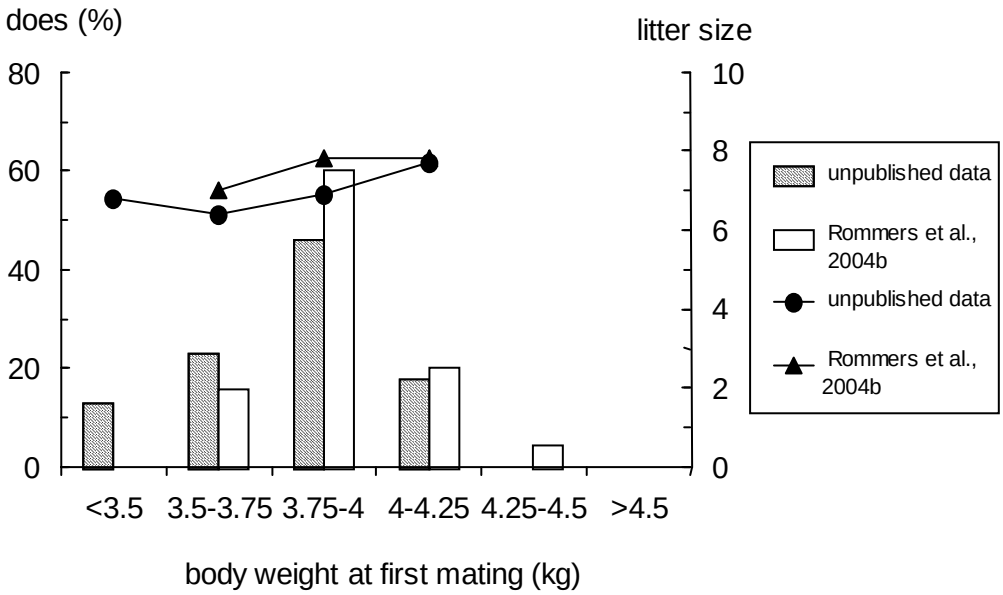

Figure 4. *Relationship between body weight at first insemination at 17.5 weeks of age and litter size in the first parity for does fed restrictively during rearing based on three experiments. The distribution of does in different body weight classes is presented in bars. Average litter size is represented by lines.*

Restrictive feeding during rearing increased uniformity in body weight at first insemination. The percentage of does that reached body weight around 4 kg at first insemination was 60 and 80% for unpublished data and data presented in Rommers *et al.* (2004b), respectively. Compared to Figure 2, there was no strong relationship between body weight at first insemination and litter size. Litter size seems to plateau around approximately eight kits from 3.75 to 4 kg for does in unpublished results. Does showed a small increase until 4 to 4.25 kg was reached (Rommers *et al.*, 2004b).

It can be stated that restrictive feeding reduces variations in body weight at the first insemination. This means a considerable decrease in the number of excessively small does at 14.5 weeks of age and in the number of excessively heavy does at 17.5 weeks of age, as was seen under *ad libitum* feeding conditions. This explains the lack of a clear response on litter size in restrictive fed does.

4.3. Stillbirths

Neither the litter size in which does were raised before weaning nor fibre level in the diet after weaning had any affect on stillbirths (Rommers *et al.*, 2001a; Babile *et al.*, 1982; Xiccato *et al.*, 1999; Nizza *et al.*, 1997). Rearing strategy after weaning (Rommers *et al.*, 2004b) revealed no clear relationship between body weight at mating and stillbirths within one rearing strategy. The percentage of stillbirths varied greatly among does within treatments. A higher percentage of stillbirths was found only for heavy does (body weight >4 kg) inseminated at 14.5 weeks of age compared to small does (body weight <3.5 kg) (13.4% *vs.* 4.6%, respectively) (Rommers *et al.*, 2002). No difference in stillbirths was found between AL-14.5 and AL-17.5 (Rommers *et al.*, 2004b).

The percentage of stillbirths seemed lower for R-17.5 compared to AL-14.5 and AL-17.5 (2.3, 6.5, 13.5%, respectively; Rommers *et al.*, 2004b), but the difference was not significant. The number/percentage of litters, which had one or more stillborn kits was also determined. Fifty percent (20 litters with stillborn kits/40 litters) of the does in AL-14.5 had stillborn kits, 22.5% (9/40) in AL-17.5, and 9.1% (4/44) in R-17.5. Restrictive feeding seems to reduce the incidence of stillbirths. The percentage of does with stillborn kits seems related to the voluntary feed intake of the does in the last week before kindling (Rommers *et al.*, 2004a). In does with a low feed intake, an increased number of litters with stillborn were found. Foetal growth in rabbits is characterized by a high growth rate during the last 10 days of gestation (Xiccato, 1996). Parigi-Bini *et al.* (1992) showed that the feeding level during gestation can affect mortality at birth. They reported a lower mortality rate at birth at higher feeding levels during gestation. However, feeding a high energy diet during pregnancy tends to increase the number of stillbirths (Pascual *et al.*, 1999; Quevedo *et al.*, 2005).

The lower number of does with stillborn in the R-17.5 compared to the AL-17.5 group in our experiments could be explained by the higher feed intake during the last week of gestation that was found in R-17.5 does (137 g/d *vs.* 172 g/d for AL-17.5 and R-17.5, respectively). The lower feed intake in AL-17.5 does could be explained by the higher fat content of these animals at first insemination compared to R-17.5 does (24 *vs.* 17%, respectively) and this is in agreement with the findings of Partridge *et al.* (1986), who found higher kit mortality at birth in animals with substantial quantities of fat.

In order to minimize stillbirths, restrictive feeding during rearing is preferred, since does will then eat more throughout gestation, resulting in fewer stillbirths at kindling.

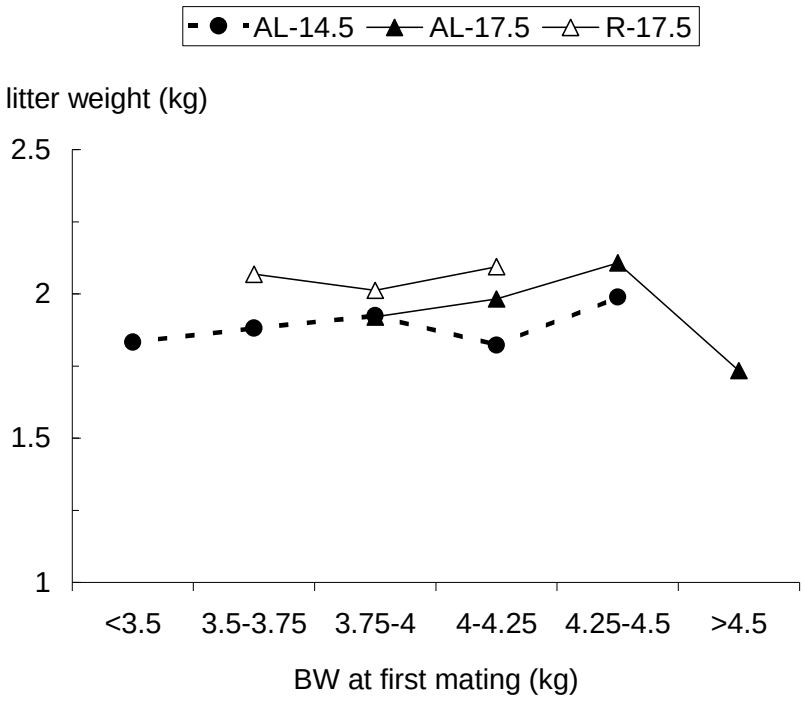

Figure 5. *Relationship between body weight at first insemination and litter weight at 16 d in the first lactation for does fed ad libitum (AL) during rearing and inseminated at 14.5 or 17.5 weeks of age, and does fed restrictively during rearing (R) and inseminated at 17.5 weeks of age.*

4.4. Milk production

The size of the litter in which does were raised before weaning did not influence milk production (Rommers *et al.*, 2001a). Fibre level in the diet after weaning tended to improve the litter weight at 21 days (2685 *vs.* 2584g) and the weight of the kits at weaning on day 35 (923.7 *vs.* 898.1 g) in the experiment of Nizza *et al.* (1997). This could be partly explained by the increased energy intake during lactation for the does raised on a fibre-rich diet. Xiccato *et al.* (1999) did not find any effect of the fibre-rich diet during rearing on feed intake during first lactation. Milk production was not affected by the fibre-rich diet and the number of kits at weaning tended to decrease.

Rommers *et al.* (2001a, 2001b, 2002, 2004b) used the litter weight at 16 days of lactation to estimate milk production in all experiments, since the kits start to consume solid food from 17 days onwards (personal observation). The relationship between litter weight at 16 days and body weight at first insemination for the different rearing strategies is presented in Figure 5. In AL-14.5 does, milk production is independent of body weight at first insemination and varies between 1.8 and 2 kg. At 17.5 weeks of age, in does fed *ad libitum*, milk production slightly increases when does are heavier at first insemination, but the level of milk production is similar to AL-14.5 does. At 14.5 weeks of age, there were no does with a body weight of more then 4.5 kg.

In AL-17.5 does heavier than 4.5 kg at first insemination, milk production drops to approximately 1.7 kg ($P<0.01$). This could be related to the reduced feed intake during first lactation. Average feed intake during the first 16 days of lactation decreased by 10% in does weighing more than 4.5 kg compared with does weighing 4.25 to 4.5 kg (unpublished data).

Does inseminated at 17.5 weeks of age and fed restrictively during rearing had an increased litter weight at 16 days of lactation compared to AL-14.5 and AL-17.5 (approximately +200 g), independent of body weight at insemination. Restrictively fed does inseminated at 17.5 weeks of age had an increased feed intake during the first gestation (+20%) and first lactation (+10%) compared to AL-17.5 does. The extra available energy is most likely used for milk production.

In lactating does, the R-17.5 group ate approximately 10% less food than those in AL-14.5 during the first 16 days of lactation. However, AL-14.5 gained in weight in the first gestation as well as in the first lactation period. This suggests that AL-14.5 still have a “drive for growth”, because of their physiological immaturity at the first insemination. Competition for nutrients between body growth and production must have occurred, resulting in smaller litters and lower milk production. This was not the case of the R-17.5 does, and explains the increased milk production in R-17.5 compared to AL-14.5 does, as shown in Figure 4.

Higher milk production will result in greater kit growth and/or lower kit mortality before weaning. The best productive performances were found in R-17.5 does (Rommers *et al.*, 2004b).

As we have seen, under *ad libitum* feeding conditions during rearing, milk production during first lactation increases slightly with body weight at first insemination until does are very heavy (> 4.5 kg). Milk production is not influenced by age at first insemination, but depends on the feeding strategy during rearing. The best performances were found in does fed restrictively during rearing, probably because they eat well during lactation (as compared to AL-17.5 does) and give less priority to body growth (compared to AL-14.5 does).

4.5. Culling rate of does

It was hypothesised that rearing strategies could prolong the reproductive lifespan of does, as the result of an improved body development or an improved feed intake and/or efficient energy utilization. However, in most studies the culling rate of does was not reported. According to the studies of Rommers *et al.* (2001b, 2002, 2004b), the culling rate of does in the first three parities was not affected by rearing strategies, being 30.4%, 24.4%, and 26.7% for AL-14.5, AL-17.5, and R-17.5, respectively.

One explanation for the absence of any effect of rearing strategy on culling rate might be that the number of does per treatment was too low. Due to practical limitations, it was not possible to include larger numbers of animals per treatment in our experiments.

Another explanation might be that the applied rearing strategies had no long-term effects on body weight development and feed intake in the first two or three parities. In all experiments, in fact, the effects of rearing strategies were limited to the first parity. In the second and third parities, no substantial effects of rearing strategies on body growth, feed intake, and reproductive performance were found.

Although rearing strategies affected body weight and body composition at first insemination, they gave no substantial benefits in terms of body weight development, body composition and feed intake over two or three parities. Heavy does at first insemination (AL-17.5) lost substantial body weight during the first gestation. *Ad libitum* fed does inseminated at 14.5 weeks of age showed substantial growth during first gestation that resulted in decreased reproductive performance in the first parity. Feed intake of R-17.5 does was improved in the first gestation and lactation period. However, the extra energy seems to have been used to improve

reproductive performance in the first parity rather than energy balance, which has also been reported by Xiccato (1996). This preference for reproduction makes it difficult to improve the energy balance during first lactations and the reproductive lifespan of rabbit does.

5. Practical implications

The results discussed in this chapter indicate that young does should have reached an optimum body weight (around 4 kg, which averages 85 to 90% of mature body weight in our studies) to improve litter size. With the current rearing strategy, this body weight is often not reached at 14.5 weeks of age. By establishing a threshold for body weight before does can be inseminated for the first time, litter size can be improved. In a cycled reproduction system, restrictive feeding can be used to increase the number of does with optimum body weight, which have to be inseminated on the same day.

Restrictive feeding during rearing involves postponing the first insemination by approximately three weeks. Besides an increase in uniformity of body weight and consequent improvement in litter size, restrictive feeding during rearing also results in improved milk production and increased weaning weight of the kits at the end of the first lactation.

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1.4. Developments in the investigation of rabbit semen and buck management

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1. Introduction

In Europe, rabbit Artificial Insemination (AI) is widely employed in rabbit farms for commercial and operational reasons (IRRG, 2005). At the same time, this diffusion has contributed to improve the knowledge both of the doe and buck physiology as well as that of the spermatozoa metabolism.

Many factors affect seminal traits (IRRG, 2005) and thus it is therefore crucial to define suitable protocols to improve spermatozoa characteristics and their fertility (Castellini and Lattaioli, 1999; Brun *et al.*, 2002b).

The aim of this chapter is to furnish the state of up-to-date information on rabbit semen and buck management focused on the following topics:

1. The spermatozoa and seminal fractions;
2. Factors affecting sperm production;
3. Limits and future prospects of semen evaluation;
4. Cryopreservation of semen;
5. Semen as a model for toxicological or metabolic studies.

2. The spermatozoa and seminal fractions

Spermatozoa undergo morphological and functional changes during differentiation, epididymal maturation and transit in the female tract. During the development in the testicle, the cytoplasm is drastically reorganised into a cell made up of a head and a tail. The different cell components are: the acrosome, localised at the top of the head, including several hydrolytic enzymes involved in the fertilisation process; the nucleus, containing DNA strictly linked with protamin proteins to block DNA functions, and the different sections of the tail, responsible for the motility.

These modifications as well as the presence of different seminal fractions affect physiological events such as the capacitation and Acrosome Reaction (AR), which are crucial for the fertilizing ability of spermatozoa.

2.1. Seminal Plasma (SP)

Seminal plasma (SP) has a complex composition adapted to the multitude of reproductive modes (Kamp *et al.*, 1996). This fluid, mainly secreted by accessory glands, provides the environment that ensures the success of fertilization.

The effect of SP on sperm survival is controversial and species-specific, and it is difficult to establish a standard action since complex interactions are involved. There is evidence to show that spermatozoa deteriorate if the concentration of SP is seriously reduced by dilution or washing (Dott *et al.*, 1979). On the other hand, other authors (Reddy *et al.*, 1979) described the adverse effect of SP on the survival of human germinal cells. However, whether beneficial or detrimental, these effects can greatly influence the behaviour of the spermatozoa.

In rabbits, the SP seems to exhibit a positive role (Castellini *et al.*, 2000b): spermatozoa suspended in TRIS-buffer, or with an extremely low content of SP, lose motility and viability within 1-3 h, while spermatozoa suspended in SP diluted up to 10-fold, maintain motility during a 6 h period. Further dilutions of SP (1/20-1/30) have no effect in the initial 2 h of storage, but thereafter cause a higher decline of motility. Kinetic parameters follow the same trend of motility rate: the highest values are in samples diluted up to 1/10, whereas a sharp decline is observed at higher dilutions.

The positive correlation between lipid oxidation and cell viability indicates that peroxidation is one of the cause of sperm deterioration during storage and that SP also enhances viability by reducing oxidation.

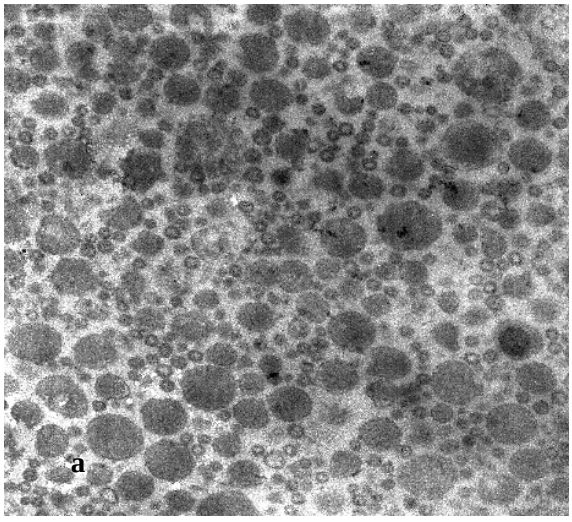
The effect of SP is due to a multitude of compounds and to the complex interactions that take place among them. Several proteins have been identified in human (Miao *et al.*, 1998; Robert and Gagnon, 1996), bovine (Bass *et al.*, 1983; Henricks *et al.*, 1998), and porcine SP (Jeng *et al.*, 1993) as the most important high molecular weight constituents with different effects on spermatozoa behaviour.

In rabbit SP, apart from a decapacitating factor (Davis and Davis, 1983), only one protein has been found and identified: a dynein-ATPase inhibitor (de Lamirande *et al.*, 1984) which prevents the initiation of motility. A few recent papers have been focused on other proteins of rabbit SP capable of modulating spermatozoa motility and various other functions (Minelli *et al.*, 2001a, b). One of these protein complexes was identified and characterised as a 150 kDa protein. This complex is believed to be a ternary complex comprising an acid-labile unit, an acid-stable binding protein (BP), and insulin-like growth factor (IGF). It is known that circulating IGFs are bound to specific binding proteins and form complexes with molecular masses of about 150 KDa (D'Ercole *et al.*, 1985). Some authors identified this complex in the male reproductive system (Lee *et al.*, 1994). Glander *et al.* (1996), showed that IGF-I and IGF-BP, contained in human SP, affect the motility of spermatozoa.

Such a protein complex also exerts an effect on the AR of rabbit spermatozoa: the addition of the IGF I-complex, to capacitated spermatozoa, acts as inducer of the AR. Results of IVF (*in vitro* fertilization) experiments showed high rates of fertilization with spermatozoa pre-treated by either Ca ionophore or IGF I-complex (Minelli *et al.*, 2001b).

2.2. Semen particles

Rabbit semen contains several droplets and vesicles of different size and origin, which play different and partially unknown roles.



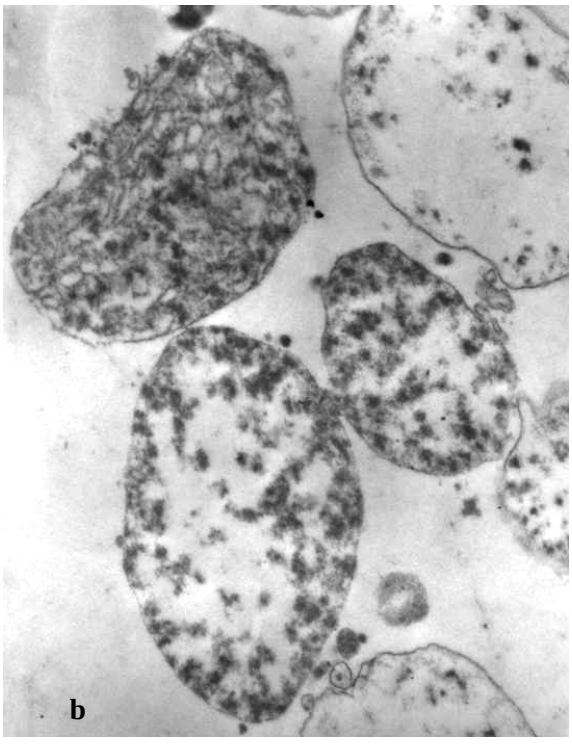


Figure 1a, b. *TEM of droplets (31,500 X) and of vesicles (102,000 X).*

In early studies, Davis (1976) showed that seminal vesicles have a capacitation/de-capacitation role and modulate AR. The role of these particles has recently been further investigated (Minelli *et al.*, 2003).

Droplets are present into a large extent in rabbit semen (Zaniboni *et al.*, 2004), whereas the presence of vesicles is limited (9.5 mg/g semen, diameter of 70 nm – Figure 1.a, b).

Since in rabbit semen these droplets have a size similar to spermatozoa (about 4 mm diameter), the simple centrifugation cannot be used to separate them (see § 3) from spermatozoa. Only Percoll® and swim-up procedures are appropriate for selecting live cells with a high degree of purity. However, the procedure of sperm separation affects the spermatozoa membrane (Glander *et al.*, 2002). Percoll®-selected spermatozoa have about half lipid content (Castellini *et al.*, 2005) with a different variety of phospholipids classes (Tamphaicitr *et al.*, 1996; Sugkraroek *et al.*, 1991) whereas swim-up safeguards provides better protection for the spermatozoa membrane (de Lamirande *et al.*, 1997) and its functional properties.

It is widely known that the cholesterol and phospholipids profiles are important in stabilising and in modulating membrane fluidity (Darin-Bennett and White, 1977). Cholesterol and also some phospholipids increase the rigidity of membranes; while phosphatidyl-choline makes it more fluid (Force *et al.*, 2001).

The other seminal fractions (droplets, vesicles and seminal plasma) showed a phospholipids /cholesterol ratio lower than that of spermatozoa. Castellini *et al.* (2005), found that rabbit seminal fractions are rich in cholesterol (Yamamoto *et al.*, 1999), which probably acts as a donor of sterols (Davis, 1976, 1980) in order to protect spermatozoa against environmental shock and premature acrosome reaction (Davis, 1981; Douard *et al.*, 2005). The presence/absence of droplets modifies the sensitivity of spermatozoa to capacitative agents: when the induction of capacitation takes place in the presence of such particles, the spermatozoa are more refractory to undergo toward AR process.

Beside of the modulation of cholesterol efflux, other authors have suggested several roles for non-sperm particles: Miodrag *et al.* (1995) found an immuno-modulatory effect of vesicles, suggesting that these may improve sperm survival in the female reproductive tract.

Minelli *et al.* (2003) showed that the seminal vesicles of rabbits are involved in AR and energy supply, being able to provide a large amount of substrate to spermatozoa 5' nucleotidase which, in turn, produces adenosine. Diadenosine compounds, although they have not been detected in seminal plasma, might be present in the female reproductive tract and the hydrolytic activity of such vesicles could increase the amount of AMP that rabbit spermatozoa will transform into extra cellular adenosine, with beneficial effects on the fertilization process.

3. Factors affecting semen production

Different factors such as year, season, frequency and type of collection and its rank, lighting programmes, buck, age and health as well as feeding strategies, can all influence sperm production. Since a recent paper (IRRG, 2005) analyzed these factors, only genetic strain and feeding will be discussed here.

3.1. Genetic component

It is known that the genetic strain can influence the semen production and the spermatozoa characteristics (Amann, 1966; Abo El-Ezz *et al.*, 1985; Dubiel *et al.* 1985). However, only recently some papers have been published that gave the results of long term studies of the semen characteristics of bucks of various genotypes (Brun et al, 2002a, Theau-Clément *et al.*, 2003, Moce *et al.*, 2005). For example, Bencheikh (1993) compared the INRA-A1077 strain (New-Zealand origin) and the INRA-A2066 (Californian origin): semen from the former collected once a week (2 ejaculates within an interval of 15 minutes) produced almost twice as much semen of better quality.

The use of crossbred males can improve sperm production: Brun *et al.* (2002a) found evidence of a significant heterosis effect for concentration (+37.5% of the parental average), mass motility (6.8 %) and the percent of motile spermatozoa (4.1 %). However, Garcia *et al.* (2004) observed a high heterosis only for the presence of distal cytoplasmic drops (35 %).

Brun *et al.* (2004), studying the effects of a divergent selection on body weight (heavy and light lines), assessed certain differences in seminal traits and hence a genetic relationship between body weight and semen production. The number of high quality spermatozoa per ejaculate (number of motile sperms/ejaculate and their ability for insemination), was higher in the light line.

Table 1 illustrates the variability of some characteristics of the semen. Repeatability, which measures the correlation between the successive performances of a buck, is generally weak. This means that the differences between males are generally low compared to the inter-male variability. Differences between bucks may arise both from genetic and environmental origin: however, when bucks of a selected strain are reared and collected according to a very strict protocol, the variability is generally lower than that obtained in less controlled conditions (Bencheikh, 1993 *vs*. Panella and Castellini, 1990).

Table 1. Repeatability of semen characteristics.

	Panella and Castellini (1990)	Bencheikh (1993)	
		Strain A1077	A2066
Volume	0.25	0.36	0.38
Concentration (spermatozoa/ml)	0.11	0.61	0.38
Spermatozoa/ejaculate	0.16	0.47	0.05
Mass motility	0.18	0.25	0.21
Motile cells (%)	0.06	0.35	0.10
PH	0.18	0.55	0.26

Current experiments aim to estimate the heritability of various semen characteristics, in order to indicate the prospects of genetic improvement by selection. Comparing semen characteristics of three rabbit strains, Theau-Clément *et al.* (2003) demonstrated that the ranking of the genetic strains varies according to objective of the study. The best strain for sperm production is not necessarily that which gives regular and optimal semen quality.

3.2. Feeding strategies

Several centres for the production of semen have been created within Europe and have permitted the development of farming practices adapted to rabbit bucks. While for some factors detailed data exist, dietary recommendations (de Blas and Wiseman 1998) are not yet available.

However, it is reasonable to assume that improvements in the quantity and quality of semen are mainly affected by the lipid and the antioxidant diet profile, which in turn influences the behaviour, and the characteristics of the spermatozoa membrane.

3.2.1 Fat

Providing of a balanced fatty acid composition seems to be very important for obtaining high quality semen. A very large amount of spermatozoa lipids are polyunsaturated (PUFA) of n-3/n-6 series (Apel-Paz *et al.*, 2003) and these fatty acids modify the membrane fluidity and its properties. The fusogenic property of spermatozoa membrane is very important during capacitation, AR and fusion with the oocyte membrane. Increasing dietary PUFA thus increments their respective levels in the membrane and improves the kinetic traits of rabbit spermatozoa (Castellini *et al.*, 2004).

3.2.2 Antioxidant

The high unsaturation of spermatozoa lipid membrane renders these cells very susceptible to peroxidation, which affects membrane structure, physiology and DNA integrity. Antioxidant protection of spermatozoa is assured by several compounds in the seminal plasma (enzymes, albumin, urate, tocopherols and ascorbic acid (Mann and Lutwak-Mann, 1981) and is partly affected by their content in the diet. Dietary PUFA supplementation therefore endangers the antioxidant equilibrium of semen, which must be restored by the administration of antioxidants.

One of the more widely used molecules is the α-tocopherol: rabbit bucks fed supra-nutritional levels of antioxidants (200 mg/kg α-tocopheryl acetate and 0.5 g/L vitamin C; Castellini *et al.*, 2000a), show a lower semen lipo-peroxidation. These trends are more pronounced in semen submitted to storage or when diets contain high PUFA levels.

Zaniboni *et al.*, (2004), showed a high level of α- and δ-tocopherol in spermatozoa followed by a progressively lower proportion in droplets and seminal plasma. The possibility that the d-isomer could be a more active antioxidant for rabbit semen is being studied.

Under field conditions, the antioxidant equilibrium is even more important due to the non-perfect hygienic conditions that could produce infection/inflammation of the reproductive apparatus, which has a negative effect on both testicle functions and semen characteristics (O'Bryan *et al.*, 2000) by affecting the biosynthesis of pro-inflammatory eicosanoids, cytokines (Knapp, 1990) and reactive oxygen species (ROS - Jones *et al.*, 1979).

4. Limits and future prospects of semen evaluation

In vitro traits give access to elementary reproduction steps permitting an understanding of complex phenomena. Unfortunately, they do not give a precise estimation of fertility since many factors (sperm transport, sperm-egg interaction, embryo growth/transport in the oviduct, embryo development) hamper successful insemination and the number of live born.

However, with *in vitro* tests, the accuracy of fertility prediction could be hypothesized. Generally, tests which include a certain level of *in vivo* situation, such as tubal AI (§ 3.2.2), should be more closely related to natural processes, which consist of numerous barriers and developmental assistances, and should permit to the elemental components of the “true fertility” to be calculated.

Among the different semen quality traits, motility determined by Computer Assisted Semen Analyis (CASA), in combination with sperm morpho-physiology, seems to provide useful information on the fertilising ability of rabbit semen (Lavara *et al.*, 2005). Therefore, efforts must be focused on rapid and objective evaluation of these parameters. It must be remembered that rabbit semen has peculiar characteristics: together with spermatozoa, it contains other components such as droplets and vesicles (see § 1.2). Moreover, when frozen-thawed semen is analysed, granules of the egg yolk used as freezing extender (see § 4.2) are present.

The estimation of sperm quality can therefore be biased by these components. In particular, when CASA systems are used, some of these components could be counted as static cells or, when the fluorimetric method is used for measuring sperm cell viability, these components could interfere with fluorescence readings. The objective evaluation of rabbit semen quality therefore requires a correct setting of the instruments.

4.1. Application of CASA systems to rabbit semen analysis

CASA systems are commonly used for the assessment of sperm motility in different species (Verstegen *et al.*, 2002). They allow the evaluation of several of the characteristics of motility, including average path velocity (VAP, mm/sec), curvilinear velocity (VCL, mm/sec); straight-line velocity (VSL, mm/sec), linearity (%LIN=VSL/VCL), straightness (%STR=VSL/VAP), amplitude of lateral head displacement (ALH average sperm track width, mm). Progressive motility is also calculated as the percentage of spermatozoa with VAP greater then 40 mm/sec and straightness greater than 80 %, whereas the percentage of rapid motility is the proportion of cells with a VAP greater then 40 mm/sec. Figure 2 shows the track path of a sperm (dotted line) and the tracking parameters.

The standardisation of the evaluation of semen samples was one of the primary reasons for the development of the CASA systems. It is generally agreed that, when the parameter settings of CASA and the operational procedure for the analysis of the sample are defined, the reproducibility of the CASA results is clearly better than that of the visual estimation of motility (Holt *et al.*, 1994). However, the evaluation of CASA results is problematic since the comparability of different systems is not satisfactory (Krause and Viethen, 1999).

Comparisons among different systems form the basis of quality assessment. Even in human andrology, a comparison has not yet been routinely established. Guidelines on the application of CASA technology into the analysis of mammalian spermatozoa have been published by the Andrology Special Interest Group (ESHRE, 1998). Among its general recommendations, internal and external quality controls are considered fundamental requirements for any CASA system. As an internal quality control procedure, it has been suggested that prepared videotapes be used when the machine is switched on for use and each time after the operating parameters have been changed to ensure that all motile spermatozoa are being identified using the current set up parameters.

External quality control requires the delivery of samples to several laboratories. For motility assessment, semen samples must be frozen following standard procedures and shipped in liquid nitrogen. To date, no satisfactory example of such an external ring study, in particular regarding CASA measurements, has been reported. Within the framework of the International Rabbit Reproduction Group (IRRG) and COST Action 848,

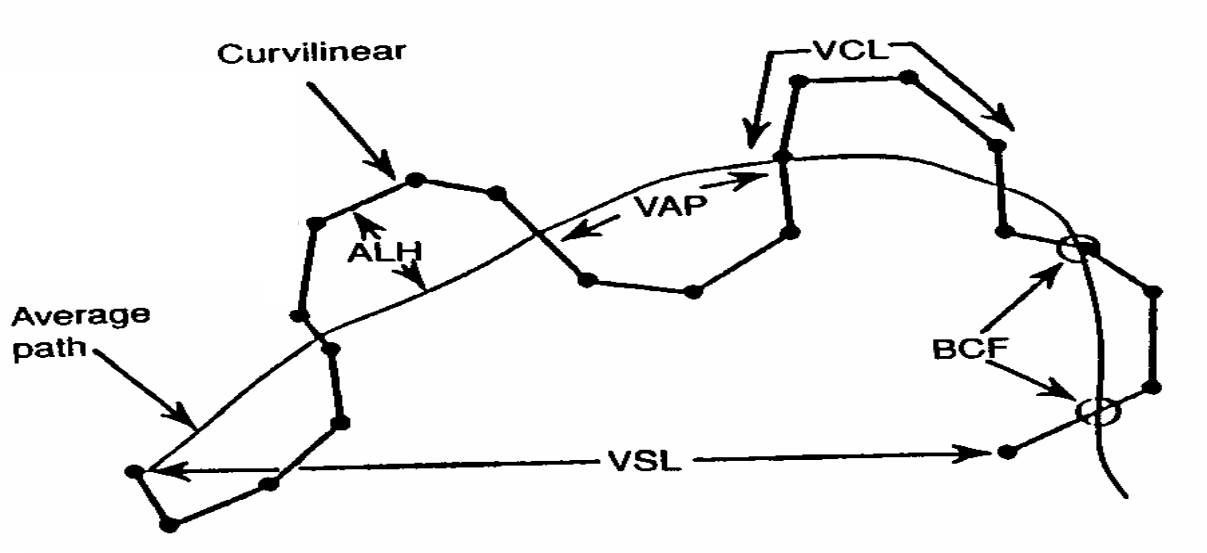


Figure 2. *Exemple of the track path of a sperm (dotted line) and the tracking parameters.*

a ring test among different systems has been developed. The systems tested were Microptic (SCA®2002), Hobson Tracker (HTS), and Hamilton Thorn Motility Analyser (HTMA). Each CASA system was located in a different laboratory and the same biological material was compared. The outcomes of the analyses performed by the three CASA systems on frozen-thawed rabbit semen were submitted to statistical analysis. The amplitude of the coefficients of variation of the different traits evaluated by the three systems was similar and high, probably responsible due to the low correlation between parameters. The presence of many egg yolk granules, as well as a high "straw effect", presumably interfered with the estimation. To avoid these limitations the possibility of comparing the systems using refrigerated semen is being studied.

3.2. Alternative systems of semen evaluation

4.1.1 Sperm viability

Together with motility and morphology, sperm viability is an important parameter in the evaluation of semen quality evaluation. Sperm viability can be evaluated by using different procedures, mainly based on the dye exclusion principle: dyes penetrate in dead cells, and stain them, while they are excluded from intact-live cells. Stained cells can be evaluated by microscopic or fluorometric methods. In particular, flow cytometry allows the rapid assessment of spermatozoa viability based on a large number of cells (Garner *et al.*, 1986). However, both the methods have certain limitations in their application into routine sperm evaluation: microscopic methods are subjective, and both labour and time consuming, while flow cytometry is an expensive process.

An alternative system for the assessment of sperm viability is the fluorometric ethidium bromide exclusion procedure. Unfortunately, rabbit semen does not allow for reliable fluorescence reading; possibly due to the interference of the granules, characteristic of rabbit seminal plasma, was suggested as its cause (Gliozzi *et al.*, 2003). Rabbit semen should therefore be washed by several methodologies in order to remove the granules from the sperm suspension. The complete absence of granules has not been obtained, however the best result being provided by the 35/70% Percollo® density gradient. The working sperm concentration range giving a constant proportion of damaged cells for Percollo® washed semen was 4-16 x 10^6. Authors concluded that the ethidium bromide exclusion procedure is suitable for washed rabbit spermatozoa with certain limits for diluted rabbit semen.

4.1.2 Tubal insemination

In vitro study of early reproduction events has become a great challenge since it offers access to multiple physio-pathological aspects for many research disciplines. However, the gain of information *in vitro* does not always mean real progress in knowledge of reproduction. To overcome such barriers it is suggested that research projects use a model such as tubal insemination to increase the predictability of *in vitro* observed effects on rabbit reproduction performance.

Viudes-de-Castro *et al.* (2005) studied an *in vitro* model for predicting *in vivo* reproductive performance without addressing the problem of the relationship between *in vitro* and *in vivo* results. Besenfelder (2005), in agreement with this strategy, studied a similar model focused on the fertility traits at the beginning of embryogenesis. Oocyte fertilisation was performed *in vivo* with *in vitro* selected spermatozoa inseminated in different female tracts prior to embryo collection to limit the effects exerted by the female site. Semen was collected and used for three different insemination groups as shown in Table 2.

Table 2. Semen preparation for different insemination techniques.

Treatment	Semen	AI ⇧ - ovulation induction ⇩ interval -4-6 0 4-6 Hours	Via	Fertilized ova (%)
Group I	Ejaculate + TRIS-buffer	⇧ ⇩	Vagina	60-98
Group II	Ejaculate	⇧ ⇩ ⇩ ⇧	Tubal insemination “	80-100 0
Group III	Swim-up selected spermatozoa	⇧ ⇩ ⇩ ⇧	“ “	0 75-90

Embryo collection was performed 20 h after the induction of ovulation. The number of fertilised ova recovered by conventional AI (Group I) ranged between 60 and 98 %. However, there was a difference in the results of Group II and III. Nearly all the oocytes were fertilised in the Group II, when AI occurred before the induction of ovulation. Moreover, many spermatozoa were attached to the Zona pellucida, a phenomenon that was not observed in Group I. Regarding the number of pronuclei, there was no evidence of polyspermia. On the other hand, the tubal insemination after the induction of ovulation did not result in oocyte fertilisation.

The swim-up selected spermatozoa for tubal insemination (Group III) showed the reverse effects: only insemination immediately before the expected ovulation time delivered embryos comparable to those obtained in Group II. Such results could perhaps be explained by hypothesising the different capacitation status of spermatozoa: native spermatozoa are protected by the extra-cellular texture and viscosity of the ejaculate whereas swim-up spermatozoa were less protected by SP and already capacitated. Tubal insemination with native semen just before ovulation did not fulfil the necessary time span for capacitation and consequently, the oocytes were not fertilised.

These preliminary findings could be considered as the basis for new lines of study such as the effect of tubal dynamics on sperm distribution, polyspermia, and the inhibitor effects of various ejaculate components. Moreover, semen of minor quality as well low quality semen and low doses can be used to examine the limiting effects on fertilisation, which may be important for animal breeding strategies based upon selection of individual lines and bucks. The limited success of subvital cryopreserved semen may also attract attention.

Access to *in vivo* studies, which minimize the time gap between treatment and traits, provides also valuable information for the optimal correlation between factors and the corresponding effects.

5. Cryopreservation of semen

Although the first successful cryopreservation of domestic animals spermatozoa dates back to the 1950s (Smith and Polge, 1950), mainly frozen bull spermatozoa is the most frequently used on a large commercial scale. Several protocols have been applied to freeze rabbit semen (Sawada and Chang, 1964; O'Shea and Wales, 1969) and sporadically good fertility rates were obtained, although litter size was always reduced (Stranzinger et al, 1971; Andrieu and Courot, 1976; Weitze *et al.*, 1976; Hanada and Nagase, 1980; Theau-Clément and Roustan, 1982).

The insemination of frozen semen in sexually receptive does achieves better results (Theau-Clément *et al.*, 1996; 79.0% fertility and 9.1. live born), while for the non-receptive does the results are very poor (20.3% fertility).

In a very few experiments, no differences were observed between the reproductive performance of fresh and frozen semen (Parrish and Foote, 1986; Chen *et al.*, 1989; Viudes de Castro and Vicente, 1996); however, none of these protocols has been demonstrated to be commercially competitive at commercial level.

5.1. The main reasons for impaired results

Many authors assessed the damages caused to the spermatozoa by freezing and thawing in different species. This can lead to a lower lifespan of the spermatozoa and to impaired transport in the female reproductive tract. This structural and physiological damages partly explains the inferior reproductive performance obtained with frozen semen (Maurer *et al.*, 1976; Parks and Graham, 1992; Watson, 2000; Mocé and Vicente, 2002).

One of the main reasons for the reduction in motility and lifespan of the spermatozoa is the alteration of the membranes and organelles that occurs during the freezing-thawing process (Figure 4). It is well known that spermatozoa are subjected to various stresses while being frozen, and that freezing has to be slow enough to prevent intracellular ice formation (which is lethal) but fast enough to permit the freezing of extra-cellular water to minimize the harmful effects of prolonged exposure to high salt concentrations.

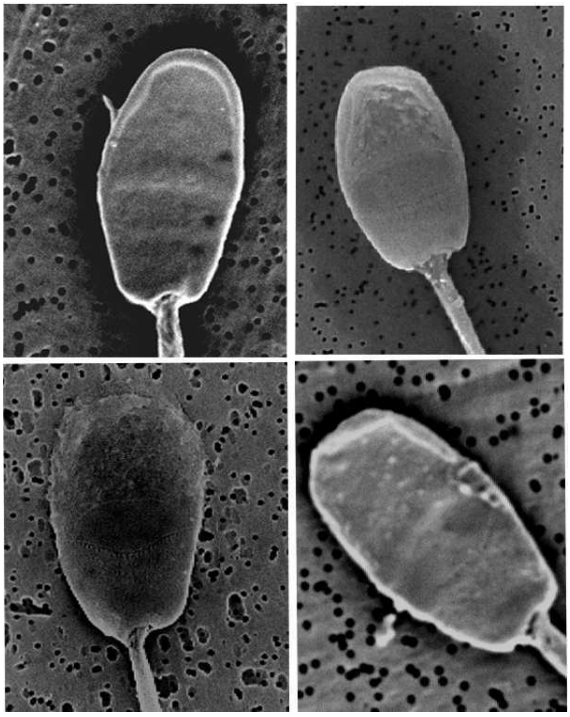


Figure 4. *Membrane injuries in frozen rabbit spermatozoa: (a) undamaged spermatozoa (b, c, d) spermatozoa with several degrees of damaged membrane.*

Even the problem of the movement of spermatozoa transport along the female reproductive tract is related to the alteration of motility patterns and to the spermatozoa viability. Viudes de Castro *et al.* (2005) have observed that the cryopreservation affected the state of the acrosome and certain motility traits, reducing curvilinear and path velocity, and ALH. In comparison to the kinetic traits of fresh semen, the same authors observed lower values immediately after thawing and a fast decline in motility rate after 4 h of co-incubation; as a result, only part of the frozen spermatozoa in the cumulus-oocyte complex were still capable of movement.

Early papers (Murdoch and O´Shea, 1973) detected fewer sperm in the oviducts of females inseminated with frozen spermatozoa along with less accessory spermatozoa in oocytes (Parrish and Foote, 1986).

In addition, the environment of the female reproductive tract greatly affects the transport (Murdoch and O´Shea, 1973), fecundation and embryo survival (Overstreet and Cooper, 1979) and for such defective spermatozoa the losses are probably higher. Thus, only some spermatozoa reach the oviducts of the doe, where they would remain alive for a very short time.

The reduced longevity of frozen spermatozoa has been related to the generation of ROS during cryopreservation (Upreti *et al.*, 1998; Fraser *et al.*, 1995). Moderate level of ROS starts the capacitation process, but severe antioxidant unbalance is retained detrimental for sperm survival (Medeiros *et al.*, 2002). Excessive production of ROS, in the form of anion superoxide, peroxide hydrogen, and nitric oxide, starts a cascade of events which could lead to premature capacitation-like phenomena, destabilization of spermatozoa membranes and AR (Pérez *et al.*, 1996; Watson, 2000; Viudes de Castro *et al.*, 2005).

If undamaged frozen spermatozoa reach the oviduct with a good capacitation pattern, a normal oocyte penetration could occur. Even in this case, the development of a regular zygote can fail to occur. It has been observed that freezing-thawing changes the condensation of DNA (Hamamah *et al.*, 1990). In bulls, boars, and stallions (Ballachey *et al.*, 1987; Evenson *et al.*, 1994, Love and Kenney, 1998), this susceptibility of spermatozoa DNA toward denaturalisation has been negatively correlated to fertility. Preliminary results also indicate the influence of DNA heterogeneity in determining the pregnancy rate in rabbits (Pizzi *et al.*, 1996).

5.2. Technical aspects of freezing rabbit spermatozoa

The qualitative characteristics of spermatozoa after thawing are not only determined by the male effect and sperm quality before freezing. Many other factors interact to play an important role: the medium (the cryoprotectant and its concentration), dilution rate, type of package and both the freezing and thawing rates.

5.2.1 Freezing medium

Generally, an organic buffer such as TRIS and anhydrous citric acid supplemented with fructose or glucose is used to support the physiological activities of spermatozoa. However, to reduce spermatozoa cryo-injuries, different cryoprotectants can be added to the organic buffer, so, the rabbit freezing medium generally contains egg yolk (10-20% V/V) and dimethylsulphoxide (DMSO), glycerol or acetamide as cryoprotectants (Stranzinger *et al.*, 1971; Weitze *et al.*, 1976; Hanada and Nagase, 1980; Chen *et al.*, 1989; Arriola and Foote, 2001). Usually, the use of 1-1.5 M of DMSO provides 40-50% motility and 30-70% acrosome integrity (Weitze *et al.*, 1976; Castellini *et al.*, 1992). Acetamide achieves a similar level of motility and acrosome integrity (Chen *et al.*, 1989).

Other studies comparing DMSO and acetamide provided contradictory results: Castellini *et al.* (1992) obtained a higher post-thawed sperm motility rate higher for DMSO than acetamide, while the contrary was observed by Dalimata and Graham (1997) which showed that the combination of acetamide and non-permeating cryoprotectants (threalose and methyl cellulose) was more effective in preserving cells with an undamaged acrosome.

The addition of non-permeable cryoprotectants to stabilize the cell membranes concurrently reinforce the antioxidant defenses and should permit the design of new strategies for cell cryopreservation (Crowe *et al.*, 2001; Oliver *et al.*, 2001). Such a strategy seems better suited to the complex structure of compartmental spermatozoa membranes, each of which is important for the spermatozoa function.

Viudes de Castro and Vicente (1996) developed a freezing medium without egg yolk, characterized by a high level of DMSO (1.75 M) and the use of 0.05 M sucrose, which offered a good protection to acrosomal membranes.

5.2.2 Dilution rate

When fresh semen is subjected to high dilution rates or SP is totally removed, a destabilization of membranes is produced, which leads to capacitation-like phenomena and to a reduction in the lifespan of spermatozoa. In the cryopreservation process, dilution rate, freezing medium and SP (see § 1.1) can be major factors in the prevention of mechanical injuries and neutralizing ROS (Bilodeau *et al.*, 2002).

The semen dilution modulates the activity of plasma proteins, fatty acids and the ionic balance, and there may even be hyperosmotic changes caused by freezing medium to be considered. High dilution rate or the deprivation of SP before freezing affects the results obtained by artificial insemination. When

SP was totally removed, neither the insemination with a high number of spermatozoa number (100 millions per dose) nor the addition of seminal plasma after freezing improved the reproductive results (Vicente 2005, data not published).

In conclusion, rabbit semen should be frozen at low dilution rates (about 1:2 to 1:10) with a freezing medium that prevents any additional harmful effects of the freezing-thawing process.

5.2.3 Packages

Semen has been frozen in plastic straws or in pellets; similar results for motility and normal apical ridge rates were observed in spermatozoa which had been frozen in straws (0.25 or 0.5 mL, Weitze *et al.*, 1976; Mocé *et al.*, 2003a) or in pellets (Weitze *et al.*, 1976; Awad *et al.*, 2000), but pregnancy rate and prolificacy were slightly better when straws were used. Recently, rabbit semen has been successfully frozen in 2 mL plastic straws after slow, directional freezing (Si *et al.*, 2006).

5.3. Freezing and thawing rates

Optimal freezing and thawing rates depend on the package and the extenders used. Usually, semen diluted with DMSO-egg yolk-extender or acetamide-egg yolk-extender, and packaged in plastic straws (0.5 mL) is cooled for 2 to 5 hours at 5 ºC before freezing in vapour nitrogen for 10-15 minutes before being plunged into liquid nitrogen. Thawing is performed in a water bath at 37 ºC to 50 ºC for 20 seconds to 2 minutes (Andrieu and Courot, 1976; Maurer *et al.*, 1976; Chen *et al.*, 1989; Theau-Clément *et al.*, 1996; Viudes de Castro *et al.*, 2005).

Vicente and Viudes de Castro (1996), developed a rapid protocol to freeze rabbit sperm (30 minutes at 5 ºC and 15 minutes at –30 ºC, using straws of 0.25 mL), using an extender without egg yolk and supplemented with 0.05 M of sucrose and 1.75 M of DMSO on sexually receptive does. The results obtained in both fertility and prolificacy were comparable to those obtained with fresh sperm (fertility rate: 81% *vs.* 79% for fresh and frozen sperm, respectively, and 8.1 *vs.* 8.0 total born for fresh and frozen sperm, respectively).

Subsequent attempts to inseminate does with spermatozoa processed according to this protocol in a commercial farm, and using animals belonging to different genetic strains, did not give the expected results (50% of fertility rate and 6.9 total born, Mocé *et al.*, 2003a).

Chen and Foote (1994), using a programmable freezer, demonstrated that mechanical seeding of extended semen at -6 ºC and freezing -15 ºC/min (from -6 ºC to -100 ºC) before plunging into liquid nitrogen substantially improved post-thaw progressive motility of spermatozoa.

The successful freezing of rabbit semen should permit to ensure the absence of any health risk, to optimize the use of semen from males of high genetic value, for production, conservation, and genetic programmes. Nevertheless, several restrictions on the use of frozen semen in rabbit farms still persist and much remains to be done in order to exploit the great opportunities offered by cryopreservation of semen.

5.4. Freezing ability in relation to genetic effect

In many species, differences in the fertilizing ability of spermatozoa from different males have been observed. Differences in the SP composition have been suggested, among others, as the possible cause of the variation in fertility rate between males. Killian *et al.* (1993) observed that bull SP contained different protein patterns, depending on the fertility of the male; Frazer and Bucci (1996), and Brandon *et al.* (1999) observed differences between stallions in the relative amount of different SP proteins.

Courtens *et al.* (1994), found differences between rabbit strains in the percentage of decondensed DNA negatively related to litter size when working with fresh semen. Freezing could increase the decondensed DNA rate differentially among different genotypes, affecting the possibilities of successful freezing.

It is interesting to note the different fertilizing ability of spermatozoa from different rabbit strains, which had been frozen by the same protocol (Mocé *et al.*, 2003a). Whereas for maternal lines fertility, rates of 80 or 85% were observed, results were worse for the growth line (50%). Preliminary trials showed the same trend (50% *vs.* 21% fertility rate and 8.0 *vs.* 5.1 total born respectively, for maternal and growth males). The differences in response to sperm cryopreservation are not yet explained and could be linked to variations in seminal plasma or in sperm membrane. Viudes de Castro *et al.* (2004), observed differences in two proteins of seminal plasma (14 KDa and 41 KDa) between the maternal and the growth line.

6. Rabbit semen as model for toxicological or metabolic studies

It is widely known that exposure to certain chemical compounds can alter semen quality and both animal and human fertility. Since the incidence of human infertility in industrialized countries has increased over the past decades, the assessment of the risk to the human reproductive system associated with exposure to these compounds -due to the pollution- is of major concern.

Rats and mice are widely used in toxicological studies, because of the massive amount of information available on their development and functions, along with their responses to many different toxicants. The main physiological traits of rabbits have also been studied, both as a laboratory animal and as meat producer in many countries

(especially in Europe). The rabbit is the smallest and least expensive laboratory animal model in which almost all the reproductive and toxicological endpoints of humans can be measured. Rabbit semen can be easily collected by an artificial vagina and the fertility of sperm tested (Foote and Carney, 2000).

Apart from *in vivo* models, a very useful method could be the use of spermatozoon as a toxicological target. The mature spermatozoon is a cell that eliminates many of the normal biological functions and retains those providing the motility (the tail) and the fertilisation ability (the acrosome see § 1.1). Damages to one of these structures is easily detectable since the ejaculated spermatozoon is unable to activate a complete repairing process.

Following these considerations, Kamp *et al.*, (1996) used spermatozoon for metabolic studies and D'Cruz *et al.* (2000) for toxicological studies. According to such assumptions, some authors (Young *et al.*, 1992; Foote, 2002) suggest the use of rabbit spermatozoa as a model for toxicological studies and propose motion-based indices as toxicological endpoints. For various chemicals, the motion-based endpoints of spermatozoa, the endpoint of the neutral red *in vitro* assay for cytotoxicity, and LD50 values are the same, suggesting that chemical inhibition of sperm motility may be useful for the *in vitro* assessment of chemical cytotoxicity.

It is quite evident that to develop a repeatable *in vitro* spermatozoa model for *in vitro* toxicity tests, different preliminary standardization steps are required., to reduce the variability factors (Seed *et al.*, 1996) and to increase the discriminating power of the model (Williams *et al.*, 1990).

Renieri *et al.* (2002), within a research program of the European Centre of the Validation of Alternative Methods, started to develop an *in vitro* rabbit spermatozoa model of spermiotoxicity for various metal ions. This involves the use of integrated strategies based on data derived from human and animal studies as well as *in vitro* toxicity tests. This may also lead to a reduction in the numbers of laboratory animals used in tests, which is very advantageous on both ethical and commercial grounds.

The preliminary steps for developing a suitable *in vitro* spermatozoa model should be the selection and the standardization of the model.

6.1. Rabbit semen model

The homogenization of the main management and environmental (*in vivo*) factors such as: genetic strain, collection rhythm, environmental temperature, light program, feed composition improves about two-fold the repeatability of seminal traits (see § 2.1).

6.1.1 Standardization of semen evaluation

As already mentioned, once selected the *in-vivo* model, the *in vitro* response of spermatozoa are greatly affected by handling (SP dilution, centrifugation; storage temperature, media, time, etc.) and thus the standardization of handling procedures is of great importance (Farrel *et al.*, 1996). The chelating power of various compounds present in semen or in the medium (SP, proteins, EDTA, TRIS) may affect the spermatozoa response (Renieri *et al.*, 2002) by modifying the availability of ions availability.

6.1.2 Endpoints

Motility rate and other kinetic traits (see § 3 - progressive speed - VSL, track speed - VCL, path velocity - VAP; linearity LIN, amplitude of lateral head displacement - ALH) are sensible endpoints, easily detectable and at a reasonable cost, combining in one characteristic the viability and the metabolizing activity of the spermatozoa.

Besides motility, also the ultrastructure of spermatozoa, evaluated by Scanning and Transmission Electron Microscopy (SEM and TEM) seems to be other helpful endpoints.

The zones most sensitive to metal ions (Sartini *et al.*, 2006) are the head plasma membrane and the acrosome. From the type and percentage of lesions, it is possible to distinguish the metal ions, even though certain metal/molecular species cause similar injuries. Normally, metal ions induced two different types of damage: a large hole on the cell membrane in the acrosomal region (Arsenic, Chrome and Cadmium) or/and cell membrane vesicolation (Platinum, Silver and Mercury).

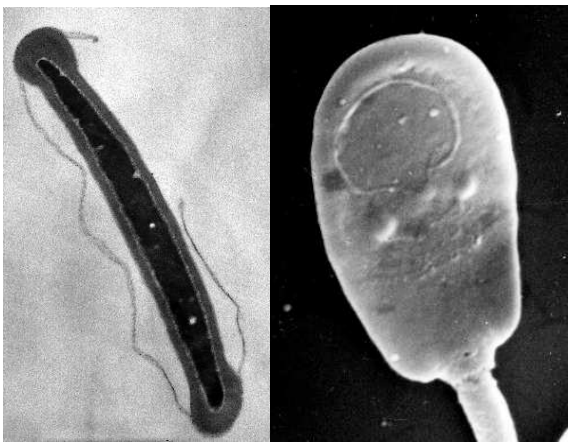


Figure 4a, b. *TEM (31,500) and SEM (x10,000) sections of the $(C_6H_5)_4AsCl \times H_2O$ 10^{-4} M spermatozoa sample*.

As an example, the TEM and SEM figures resulting from the incubation of 10^{-4} M $(C_6H_5)4$ AsCl x H_2O for 4 hours are reported. Arsenic induces a rounded hole on the plasma membrane located in the acrosomal region (Fig. 4b, SEM) with broken plasma membrane detached from the nucleus (Fig. 4a, TEM). Silver provokes membrane vesiculation on the sperm head (Fig. 5.b, SEM) with the plasma membrane completely destroyed (Fig. 5a, TEM).

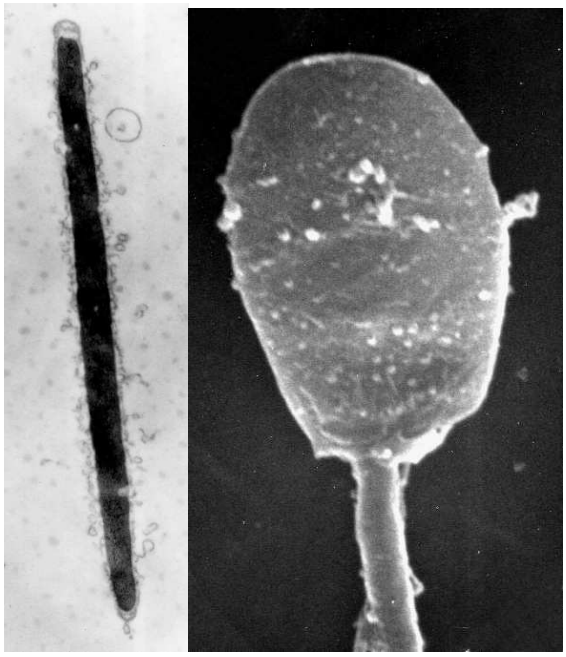


Fig. 5.a, b: TEM (x 30,000) and SEM (x 10,000) sections of the $Ag(Na)NO_3$ 10^{-4} M spermatozoa sample.

On the basis of the data obtained from the different experiments, it seems reasonable to consider the rabbit spermatozoa as a sensitive target for detecting different toxins and this line of investigation is thus capable of further development.

7. Conclusion

Rabbit production systems are associated with the successful implementation of reproductive technologies and a thorough knowledge of the mechanism of reproduction would have a beneficial effect on the rabbit industry. In particular, knowledge of the sperm metabolism certainly improves the predictor criteria of reproductive performances and the technique of semen preservation.

At the same time, these studies suggest rabbits as a very attractive and promising model for other species (both animals, and humans) due to their body size and reproductive efficiency.

The use of rabbits in toxicology studies and the implementation of reproductive technologies need a standardisation of the evaluation of semen quality. In this context, the application of new methodologies for semen evaluation represents an important step. Within the framework of COST Action 848, the principal CASA systems used for the analysis of rabbit semen were compared, thus facilitating progress in the standardisation of the evaluation of rabbit semen.

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Chapter 2

HOUSING OF RABBITS IN CONFORMITY WITH ANIMAL WELFARE AND PROTECTION CRITERIA

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In intensive rabbit farming systems, both females and fatteners are housed in small cages with wire nets or slatted floors. Growing public concern for the welfare of animals is observed under such housing conditions.

In a first subchapter the main welfare indicators are described. Besides health status parameters, behaviour is a key welfare indicator. Therefore, in several subchapters specific behaviour is presented, from the kits to the growing and breeding rabbits. In spite of many years of domestication, the behavioural repertoire seems very similar to its wild counterpart.

Females are housed individually, although they are social animals. In subchapter 7, attempts to develop a commercial group housing system are presented. Another way to house rabbits more in conformity with welfare is to enrich the environment. Both for does (e.g. platform) and fatteners several attempts have been made and are reviewed. Finally, national and European space allowances concerning housing of rabbits are discussed. Apart from laboratory animals, there are no common European guidelines or recommendations with regard to the housing and transport of rabbits.

2.1. Welfare indicators

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1. Introduction

Breeding rabbits are usually kept in intensive husbandry systems, mainly in cages with wire nets or slatted floors. The housing of reproducing does is related to behavioural, hygienic, environmental and welfare aspects. The meaning of "welfare" has been defined by many authors, and many definitions of the term have been given (see literature in Verga, 2000).

In breeding rabbits, guidelines for welfare should take into account the "five freedoms" (FAWC, 1991), i.e. freedom from hunger and thirst, from an inadequate environment, from pain, injuries and distress, from "fear" and from the impossibility of expressing the "normal" behavioural repertoire. According to the literature, the same welfare indicators used for other farm animals may also be used on rabbits (Morisse and Maurice, 1994; Verga, 2000). The main welfare indicators are listed in Table 1.

2. Welfare indicators

No or low (unavoidable) mortality is the most important welfare criteria. Health status of rabbits is also a key welfare parameter. The morbidity rate including infectious factorial diseases and injuries should be low and unavoidable. Welfare evaluation of both rabbits and rabbitries should consider both physiology and behaviour. Hormone levels, heart rate variation and immune reactions can be used as an indicator for the housing conditions, but should be considered only in relationship with other parameters (behaviour, morbidity). Examples are given by Verga (2000). Immune system activity (immune cell proliferation and immunoglobulins synthesis) and oxidative-antioxidative status are important indicators of the coping ability of (heat) stressed rabbits. However, in practice, these parameters cannot be measured directly in the rabbitry as special technical equipment is necessary.

Temperature is one of the most studied stressors in rabbits, especially the effects of heat stress. A hot climate may negatively affect maternal and sexual behaviour. Some research has been carried out showing the physiological effects of climate in relation to housing systems and management. The stress indicators evaluated are mainly physiological and production variables.

Table 1. Main welfare indicators for rabbits (after Verga, 2000, Hoy, 2005a, b).

1. Mortality: no or low (unavoidable) mortality
2. Morbidity: pathologies ("internal diseases", infectious factorial diseases); injuries – the morbidity should be low and unavoidable
3. Physiology: hormone levels, heart rate variation, immune reactions – the physiological parameters should be in the species-specific standard
4. Behaviour: ethogram, reaction to behavioural tests – species-specific behaviour
5. Performance (production): growth, feed conversion, fertility rate – the performance should be on a good ("normal") level

Heat stress reduces feed consumption and may affect certain physiological variables, such as total proteins, glucose and total volatile fatty acid concentration in the caecal content.

The presence of "abnormal behaviour" (e.g. stereotypies – Lawrence and Rushen, 1993) may indicate the existence of problems (Podberscek *et al.*, 1991), but pawing the floor or gnawing at the walls may also be considered "normal" behaviour in an inadequate environmental context (Morisse and Maurice, 1997). Other significant parameters of acute loading or stress may be evaluated by considering other aspects of behaviour, such as feed intake (Finzi *et al.*, 1986), social and maternal behaviour (Verga *et al.*, 1978, Lehmann, 1991, Verga, 1997, Morisse, 1999). Ethological observations can lead to results and assessments concerning changes or disturbances in behavioural parameters caused by inadequate environmental conditions, insofar as the "normal" behaviour is known. A review of rabbit behaviour under modern commercial production and management conditions is given by Marai and Rashwan (2003, 2004).

Parameters of performance (live weight development, feed conversion, fertility) can also be related to welfare criteria. High performance is not necessarily proof of a high standard of welfare, but low performance is an indicator of defective housing, environment or management.

Therefore, housing of rabbits in conformity with animal welfare and protection means:

- the minimum unavoidable mortality,
- freedom from physical injuries,
- good health (essential medical treatment only),
- species-specific behaviour and
- conditions for development of animals corresponding to age and sex (Swenshon, 1997, Lange, 2003, Hoy 2005a, b).

Rabbitries where injuries, pain and avoidable sufferings occur, which can be avoided with the necessary care and appropriate prophylactic measures (vaccination, medication, hygiene), are not conform to the animal protection regulations.

3. Housing requirements

The general requirements for housing of rabbits can therefore be summarised:

- no pain, no avoidable sufferings and no injuries caused by housing (floor, walls, equipment),
- protection against predators, ecto- and endoparasites,
- provision of food and water appropriate for rabbits' needs (ad lib),
- protection against adverse climatic conditions,
- removal of gases, dust and pathogenic germs from rabbits' housing,
- careful handling of animals (catching quickly and firmly, without frightening or inflicting injuries),
- removal of excrement by using perforated floors if possible (especially in intensive housing),
- periodical use of "all in all out" with cleaning and disinfection,
- enriched housing system, e.g. 2nd floor/elevated platform for does.

Investigations have shown that the doe prefers to jump on an elevated seat if this possibility exists. Selzer (2000) demonstrated that does react to attempts by kits to suck in 89.5 % of all cases by jumping onto a platform. In an unstructured concrete box, the doe only has the possibility of lying down (80.7 %) or running away (13.8 %) as a means of avoiding kits' attempts to suck. The 2nd floor is used by does as a withdrawal area and the space underneath serves the kits as a hiding place. A simple elevated platform can be made from wood, but, as hygiene is very important and the second floor is also used as a place for defecation, there is a possible risk of endoparasite infection. Further research on hygienic aspects (e.g. floor material of seat) is necessary.

The separation of the does from their own faeces is very important to prevent or to reduce the occurrence of infectious diseases. A perforated floor (not only for the elevated seat but for the whole housing system) fulfils this general requirement. A plastic slatted floor may have advantages from the welfare point of view, but problems have been detected with the durability of this material.

3.1. Single housing of rabbit does

The Standing Committee of the European Convention for the protection of animals kept for farming purposes (Table 2) proposes minimum box space and height requirements that depend on the weight of rabbit does.

Table 2. Minimal space allowance and height of the box in relation to live weight of rabbit does (proposal of Standing Committee of the European Convention for the protection of animals kept for farming purposes - 45th meeting, Strasbourg, 25 - 27 November 2003).

Live weight	Minimal space allowance	Minimal height of the box	Platform
4.5 – 5.5 kg	3600 cm^2	35 cm	no
4.5 – 5.5 kg	3000 cm^2	60 cm	yes (1000 cm^2)

The approximate values for the single housing of does with kits in "get-away-cages" with 2nd floor (intensively used breeds) can be described as follows:

- width (of the cage) = 50 cm, depth = 70 cm, height = 70 cm, second floor in 25 cm height, 50 cm wide and 30 cm deep,
- 0.35 m^2 per doe with kits (plus nest box) (or 0.45 m^2 including nest box) = 3500, 4500 cm^2 respectively,
- nest boxes in all cages for reproducing does = 35 x 35 x 30 cm with litter material,
- slatted floor or wire net for the doe and older kits.

The third dimension (the elevated seat) is more important than a larger space. Rabbits kept on a full slatted floor must have access to material for investigation and manipulation ("enrichment"). Enrichment is a characteristic of animal-friendly housing. A wooden stick has no negative impact on performances but causes fewer sterotypies (Verga *et al.*, 2004). Also, "welfare friendly" pens (plastic platform, hiding box and gnawing material) have no negative impact on performance of growing rabbits (Maertens *et al.*, 2004). The wooden stick provided as gnawing material should be hung from the roof of the box, as otherwise there is a risk of it becoming contaminated with faeces.

3.2. Group housing of rabbit does

The problems involved in the group housing of reproducing does were discussed in depth during the meeting of working group 2 of the COST action 848, which was held in Wageningen (The Netherlands) in May 2004. The following difficulties related to group housing were identified:

- high number of nest visits and behavioural disturbances,
- high kit mortality,
- difficult health control,
- higher risk of disease (e.g. coccidiosis),
- replacement of does,
- higher production costs (Ruis, 2004 personal information, Hoy, 2004, see also subchapter 2.7).

It was mentioned that good basic data is necessary for a scientifically based discussion on group housing of reproducing rabbit does. The decision reached was that it is not possible to come to conclusions concerning group housing solely on the basis of practical experiences without detailed scientific results regarding mortality, morbidity and performance. One of the problems is the mutual aggression between reproducing does. This is caused by the linear hierarchy endemic to the species-specific behaviour (see literature in Selzer, 2000). Aggressive interactions may occur if new does are introduced to a given group (Schuh *et al.*, 2003). The structure of the enclosure or pen (size, enrichment) cannot prevent aggressive interactions between does (Hoy and Schuh, 2004). In investigations of Hoy (2000) and Hoy and Schuh (2004), the enclosure measured 150 m^2 for three does and one buck and aggressive behaviour was not prevented. Cages or boxes for single housing are always necessary to isolate aggressive or sick animals. The latest results concerning group housing of reproducing does are presented in subchapter 2.7.

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2.2. Nursing behaviour of wild and domestic rabbits

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1. Frequency of nursing events

In the past the belief existed that rabbit does nurse their kits only once a day (Cross, 1951, Venge, 1963, Zarrow *et al.* , 1965, Findlay & Tallal, 1971, Hudson & Distel, 1982, Bigler, 1986, Jilge, 1994). Davis (1957) and Bernard (1962) observed two nursings a day only during the first 2 or 3 days of life. Direct visual observation was mainly used in those investigations. However, in recent years investigations using infrared video technique and time lapse recording over 24 hours a day indicated that rabbit does kept in cages, not influenced by persons or artificial light, nurse more than once a day (Seitz *et al.*, 1998, Hoy, 2000, Hoy & Selzer 2002, Selzer *et al.* 2004, Maticz *et al.* 2004)(Table 1). It was discussed whether more than one nursing a day is a species-specific behaviour or a behavioural disturbance of rabbit does kept in cages that are too small.

In the latest investigations, it was demonstrated that both wild and domestic rabbits kept in outdoor enclosures nurse their kits more than once a day. In approximately 30% (wild rabbit does), 12% (domestic rabbit does), respectively, of all the observed 24-hour intervals more than one nursing was observed. Hungarian investigations have also discovered the same tendency. Twenty-five percent of the does nursed more then once a day (Maticz *et al.*, 2001 a, b). However, Seitz (1997) has shown that the frequency of nursing (≤ 1, 1 to <2 and >2) did not influence the weaning weight of kits.

Investigations of wild and free range domestic rabbits demonstrated that most of the nursing events (84.0 % in wild rabbits and 85.8% in domestic rabbits) took place in darkness. Only a small rise in nursing activity was observed in the early morning. These results correspond with data on domestic rabbits kept in separate cages (Seitz *et al.*, 1998, Selzer, 2000). The use of different methods to study behaviour (direct visual observation with presence of observer versus infrared video technique and time lapse video recording) is the most probable explanation for the different results in the literature.

Table 1. Frequency of nursing per 24 hours period in wild and domestic rabbits kept in two free range areas (Selzer 2000).

	number of 24 hr-intervals	frequency of nursing events in 24 hr[1] $\bar{x} \pm$ SD.	number of does	frequency of nursing events in 24 hr[2] $\bar{x} \pm$ SD.
Wild rabbits	104	1.28 ± 0.54	6	1.26 ± 0.20
domestic rabbits	257	1.12 ± 0.49	8	1.16 ± 0.12
		$p < 0.05$		$p > 0.05$

[1] means on the basis of all 24 hr-intervals (n = 104, 257 respectively)
[2] means on the basis of average nursing frequency of does (n = 6, 8 respectively)

2. Nursing duration

Up to the present there has been no information available on the duration of nursing in wild rabbits. According to Zarrow *et al.* (1965), Drewett *et al.* (1982), Petersen *et al.* (1988), Seitz (1997) and Schulte (1998), the average duration of a nursing event in domestic rabbit does ranged between 3 and 3.5 minutes. The mean duration of a nursing event in wild rabbit does (on average 179 seconds) is shorter than in domestic rabbit does (Selzer 2000). Selzer (2000) also reported that small rabbit breeds nurse their kits for shorter times (approximately 192 sec) than larger pet rabbit does (up to 230 sec on average). It is possible that the milk yield of smaller breeds and wild rabbits is lower and so the duration of nursing is shorter than in larger breeds like New Zealand White and ZIKA hybrids.

Diametrically opposed frequency and duration of nursing occurs during the period of lactation, in both wild and domestic rabbits. The highest nursing frequency combined with the lowest mean duration of a nursing event takes place in the second nursing week after kindling (Seitz 1997, Schulte and Hoy 1997, Selzer 2000)(Table 2).

Table 2. Frequency and duration of nursing events in wild and domestic rabbits in relation to week of lactation (Selzer *et al.* 2000).

	Wild rabbits			Domestic rabbits		
Lactation week	Duration of nursing event (sec)	% of days with ≥2 nursing events	Frequency of nursing events/24 h	Duration of nursing event (sec)	% of days with ≥ 2 nursing events	Frequency of nursing events/24h
1	184.4 ± 30.3^{d}	21.2	1.24	229.9 ± 56.9^{c}	9.2	1.09
2	169.2 ± 35.2^{d}	44.8	1.48	200.5 ± 32.0^{c}	22.2	1.27
3	185.0 ± 42.0	34.8	1.35	205.8 ± 36.3	15.1	1.15
4	186.3 ± 21.2	10.5	0.95	211.9 ± 30.4	2.8	0.99
$\bar{x} \pm$ SD	178.5 ± 34.4^{b}		1.28^{a}	211.8 ± 41.6^{b}		1.12^{a}

Means with different letters (a, b, c, d) are significantly different ($p < .05$)

Hudson and Distel (1989) postulated a fixed time interval of 24 hours between two nursing events. However, this was for rabbits (few in number) kept in sound-isolated laboratories and is not comparable with practical conditions. In the latest works, mean time intervals of 16.5, 20.5 hours are found in wild and domestic rabbits (Selzer 2000). This corresponds to a nursing frequency higher than once a day. Seitz (1997) also reported a mean time interval between two nursings of 16.5 hours, but the individual nursing frequency per doe ranged from .8 to 2.2 nursings in 24 hours (Seitz *et al.* , 1998).

Nursing behaviour both in wild and domestic rabbits follows a circadian rhythm with a peak after midnight (3 to 6 hours after onset of dusk) in wild rabbits and in the first two hours after the beginning of dusk in domestic rabbits. Light-dark-change is a significant zeitgeber (timer) for nursing behaviour especially for domestic rabbit does. More than 25 % of the nursing events take place in the first two hours of darkness if rabbit does are kept under artificial lighting conditions (Seitz, 1997). If the light-dark-regime (12 L : 12 D) is changed by one hour (from 5 am to 5 pm till 6 am to 6 pm) the peak in nursing activity is postponed simultaneously by one hour (Seitz, 1997). The peak in nursing activity is postponed under natural lighting conditions between March/April and July from 7 pm to 10 pm soon after the beginning of dusk (Seitz *et al.* , 1998). In contrast, the morning dark-light-change under artificial lighting conditions, or the onset of dawn under natural lighting, causes no or only a slight increase in nursing activity. Using an intermittent light regime with 6 hr light : 6 hr darkness : 6 hr light : 6 hr darkness, two peaks in nursing activity were demonstrated after switching off the light twice a day (Hoy, 2000, unpublished results).

There is a delayed peak of nursing behaviour in wild rabbits compared with domestic rabbits. Wild rabbits spend the time between dawn and dusk mainly in the nest box without food and water and without the possibility of urination and defecation. They leave the nest boxes with the beginning of dusk. Selzer (2000) reported that wild rabbits start with food intake and elimination soon after leaving

the nest boxes. After this period, they nurse their kits. In contrast, domestic rabbits also eat, urinate and defecate during day time. Therefore, the light-dark-change during dusk influences the onset of nursing activity as a zeitgeber (timer) compared with the conditions under an artificial light regime.

Shortly before nursing, increased restlessness can be observed. The kits push the nest material around. One-week old wild rabbit kits react with intensive vocalization approximately 15 seconds before the mother enters the nest (Hoy, 2005). Obviously, they can feel the vibration in the tube as the mother approaches.

An increase in the number of vocalizations can be observed in the three hours before the main nursing (nocturnal). On days with more than one nursing, no increase in the number of vocalizations can be observed before the second nursing (in 24 hours) (Schuh *et al.* , 2004). It seems to be that the hungry kits are the initiators of the nocturnal nursing while the second nursing is initiated by the mother, who wakes up the kits. A possible reason for the second nursing could be the increasing intramammaric pressure causing the mother to nurse the kits twice in 24 hours (Schuh *et al.*, 2004). Selzer *et al.* (2001) found 9.0 till 20.1/24 h suckling attempts in kits, according to the type of cage (get-away *vs.* conventional), while free range kits show very rare and unsuccessful suckling attempts.

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2.3. Odour cues and pheromones in the mediation of rabbit female-offspring relations

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1. Common odour cues during the birth transition

During the last days of gestation, fetal rabbits have their nostrils open, allowing sensory exposure to the odorants passed trans-placentally from the pregnant doe's diet. Such prenatally acquired odorants are carried over into the postnatal niches, so that they attract neonatal kits. For example, kits born to does fed juniper, thyme or cumin orient preferentially to such compounds after birth (Bilkó *et al.* , 1994, Coureaud *et al.* , 2002).

When simultaneously exposed to the odours of placenta and of conspecific milk in a choice arena, rabbit newborns spend equal time in proximity to both (Coureaud *et al.*, 2002), a response interpretable in terms of sensory or motivational equivalence. Indeed, some odorants from maternal food pass parallely into amniotic fluid and colostrum (Schaal *et al.* , 2003). The behavioural impact of this perinatal chemosensory overlap has been highlighted in two ways. First, kits born to cumin-eating does more often grasp a glass-stick carrying milk from an unrelated cumin-eating female than milk from a doe fed standard food, and vice-versa for kits born to does fed standard food during pregnancy (Coureaud *et al.* , 2002). Second, cross-fostering kits between does fed the same or different diets during gestation and lactation results in groups of kits contrasted for perinatal continuity exposure (Coureaud *et al.* , 2002). Also, kits exposed to perinatal odour continuity (congruent aromas in amniotic fluid and milk) were better at obtaining milk during the first three nursings than 'discontinuous' kits. Thus, the efficiency of the kits' initial sucking performance depends in part on whether the olfactory properties of milk (or of the doe's belly) are in line with the odour background of the womb. However, neonatal sucking performance depends, as described below, on other kinds of odorants that can be learned postnatally.

2. Common odour cues acquired in the nest

The materials composing rabbit nests are olfactorily salient to newborns (Hudson *et al.*, 2003). The predominant source of attractive nest odorants comes from maternal abdominal fur or plant materials (which may be part of the female's diet). But any odorous material artificially added to the nest can become rapidly meaningful (Hudson, 1993). A notable source of significant odorants in the nest comes from the hard faecal pellets that females drop at the end of each nursing visit (Hudson *et al.* , 1996). These maternal faeces (MF) elicit a sequence of collective gathering among littermates, which actively nibble and finally consume them (Moncomble *et al.*, 2004).

3. Common odour cues acquired while suckling

Kits respond to undefined natural odour cues from the maternal body surface, but are also learners of artificial odorants painted on the mother's ventral fur and nipples. Advantage was taken of these early abilities to understand the plasticity and

developmental course of odour learning in newborns (Hudson, 1985, Kindermann *et al.*, 1994, Allingham *et al.*, 1999). These studies consisted in scenting the does' ventral fur with varied odour qualities, letting kits search and suck on her, and testing them 24 h later on dummies whose odour could be easily controlled (anesthetized doe or rabbit fur). The results were as follows:

- the range of learnable odorants is very broad (e.g., citral, camphor, or complex perfumes or aromas),
- such artificial odorants are acquired in only one odour-nursing pairing,
- this nursing-induced odour learning is time-bound, as it is effective only during the first 4 days after birth (Kindermann, *et al.*, 1994) and finally
- intra-oral stimulation linked with sucking a nipple has been suggested to be the key-reinforcing agent that engages the odour learning process (Hudson *et al.*, 2002).
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Thus, nursing-induced odour learning may constitute a process by which kits prepare optimal cues for the next suckling, actualize the evolving properties of milk and anticipate odour-based changes in the mothers' diet or other conditions. But not all active odours need to be learned.

4. Specialised signals in lactating rabbits and in rabbit milk

As in all mammals, the milk of rabbits carries varied odour cues reflecting either the mothers' individuality (e.g., diet, immunogenetic constitution, stress exposure, health state) or supra-individual information related to colony, population or species. Such species-specific odours were evidenced by presenting 'naïve' kits with secretions from unrelated female rabbits or similar compounds from females of other species, or conversely in presenting rabbit compounds to newborn of different species. When approached without contact (Coureaud and Schaal, 2000) with the abdomen of different does, the rabbit kits respond discriminatively to virgin or lactating females, the latter releasing the clearest orientation.
Kits are more reactive to the abdomen of lactating does in early, rather than late, lactation, and before rather than after nursing (Coureaud *et al.*, 2001). An efficient volatile factor thus appears to be linked with lactation, and to be released from the nipples of lactating females (Coureaud *et al.*, 2001). Müller (1978) first evidenced that rabbit milk odour is unique in eliciting the responses of newborn rabbits, as bovine, ovine, porcine and feline milk were completely unable to trigger their typical head and mouth motions.

5. A pheromone in rabbit milk

The results of different tests provide minimal criteria to elect 2MB2 (2-methyl-but-2-enal) as a pheromone. As it appears to be produced in the distal part of the mammary tract (Moncomble *et al.*, 2005), it was named after its source "Mammary Pheromone" (MP). Kits that do not react to the MP on postnatal day one have lower survival chances during the next four weeks (Coureaud *et al.* , 2000a, b), indicating that initial responsiveness to the MP may be linked with viability. The mediating factors of such a phenomenon, possibly related to perceptual or behavioural deficits in individual kits, are under scrutiny. Further, recent evidence demonstrates that the MP is a strong reinforcer engaging the instantaneous learning of any co-occurring odorant (Coureaud *et al.*, 2005). The MP may thus be a potent agent for the rapid expansion of the repertoire of behaviourally significant odour cues after birth. Finally, the MP-induced release of the typical rooting-sucking behaviour vanishes progressively at the same time as the need to suck and consume milk (Coureaud, 2001). However, adding the MP to peletted food still tends to increase intake in weanling kits (Coureaud *et al.*, 2003).

6. Early odour experience and the weaning transition

Dietary aromas to which kits had been exposed prenatally not only influence neonatal responses, but can be retained for long periods to influence solid food choice at weaning. For example, rabbit does having eaten pellets enriched with juniper or thyme flavour during pregnancy and lactation produce kits displaying preference for correspondingly odorized food at weaning (Altbäcker *et al.*, 1995). Bilkó *et al.* (1994) assessed the relative influence of odour exposure in utero, in lacto or in faeces on intake in 28-day old weanlings. They compared the selective eating responses of kits which had been previously exposed to juniper aroma either through i) amniotic fluid, milk and faecal pellets, ii) amniotic fluid and milk iii) amniotic fluid alone, iv) milk alone, v) (hard) faecal pellets alone, and vi) had never been exposed to juniper. All groups that had been in contact with the juniper odour (groups i-v) showed equally strong ingestive preference for juniper berries as compared to control kits (vi). Rabbit kits have thus the potential to acquire odours from their mother's diet at any stage of development (pre- and postnatal) and in various contexts of acquisition (in presence or absence of the doe).

The overlap in the substrates that allow the doe-to-kit transmission of olfactory cues is a way for the growing newborn to progressively encode those cues

that predominate in the foodstuffs safely selected by the mother. How mother-induced early odour experience modulates ingestion in kits and in later establishment of stable dietary preferences remains to be investigated.

7. Kit odours affect maternal behaviour

The fact that newborn kits can be easily switched between litters would suggest that farmed does do not pay much attention to the odour of their offspring, at least in the first few days postpartum. They can however be observed sniffing, and sometimes licking, their kits after birth, or thereafter right before each daily nursing. Despite occasional data here and there, the behavioural and physiological influences on rabbit females of odour cues from the nest, litter or individual young remains an area for future enquiry.

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2.4. Behaviour of kits

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1. Nest behaviour

Rabbit kits are altricial animals but they have evolved a coping strategy which allows them to survive and grow till weaning in spite of receiving little maternal care, typical behaviour in rabbit does (Mykytowicz, 1968, Hudson *et al.*, 1996). After birth they stay inside the nest huddled quietly together (Hudson *et al.* 2000), obtaining energy conservation and thermal regulation between nursing sessions. This mother-kits interaction is similar in both wild and domestic rabbits (Coureaud *et al.*, 2000). The nest has been prepared by the doe, who lines it with collected material ('straw nest') and hair from her own body ('maternal nest') (Zarrow *et al.*, 1961, Denenberg *et al.*, 1969, Canali *et al.*, 1991, Gonzalez-Mariscal *et al.*, 1994, Hudson *et al.*, 2000). The nest is covered and hidden from predators. Kits generally do not escape from nests and thus retrieving behaviour from the doe is not necessary (Denenberg *et al.*, 1969).

Rabbits start their lives as social animals, and the presence of siblings allows each kit to survive due to the increased thermal efficiency (Bautista *et al.*, 2003). Competition exists among the littermates in order to achieve milk ingestion, mainly during the first week of life: the heavier kits at birth grow faster (Seitz, 1997), and over three weeks of age stable weight hierarchies have been found (Drummond *et al.*, 2000).

After the doe leaves the nest, kits urinate and then burrow back into the nest material till the next sucking time. In the second week of age kits begin to eat the faecal pellets left by the doe inside the nest as well as nibbling at the nest material (Hudson *et al.*, 2000). They discriminate between faecal pellets from the mother and an alien doe and this affects their feed choice (Hudson and Altbacker, 1994; Hudson *et al.*, 1996). Ingesting plant material and faecal pellets in the nest may prepare the kits to digest plant foods at weaning (Hudson *et al.*, 2000).

2. Nursing behaviour

Some key stimuli attract the kits to the doe's mammary region (Hudson and Distel, 1983, Mohamed and Szendrő, 1992), and they actively search for nipples and attempt to suckle. Their behaviour is stimulated by the so-called 'nipple-search pheromone' or Mammary Pheromone (MP), produced under hormonal control (Gonzalez-Mariscal, 2004, Gonzalez-Mariscal *et al.*, 1994, Moncomble *et al* , 2005 – see also chapter 2.2.). An imprinting-like learning process may be hypothesized during the first period of life, based on the kits' olfactory reaction. The maternal pheromones are present both on the doe's body and in the milk, thus anosmic kits will starve (Hudson and Distel, 1995, Hudson *et al.*, 1996, Coureaud and Schaal, 2000). They also react to tactile and vibration stimuli (Schuh *et al.*, 2004), but not to visual until the end of the first week oflife, since their eyes open only on day nine or ten (Gottlieb, 1971).

Kits, when deprived of one nursing, anticipate the following nursing session in an apparently endogenous circadian pattern of arousal, which may occur before birth (Hudson and Distel, 1982, Escobar *et al.*, 2000, Drummond *et al.*, 2000). The identification of the nipple-search pheromone (Coureaud and Schaal, 2005) could allow kits to be reared artificially, reducing mortality due to starvation, although it may be very difficult to raise them by hand (Hudson *et al.*, 2000).

Kits can drink up to 25 % of their weight in one only nursing session and show nipple searching behaviour towards any lactating doe (Hudson *et al.*, 2000). The nipple choice is not fixed, but kits change nipples very frequently during a suckling session (Distel and Hudson, 1985). On average up to 8 % of kits do not obtain milk during one nursing event (Schulte and Hoy, 1997). This percentage was 3.1 % in small litters (litter size < 5 kits) and 9.8 %

in large litters (litter size 8 to 11 kits) (Schulte, 1998).

When kits have left the nest at the age of 12 - 15 days they will try to suckle from alien lactating does, and these may nurse alien kits (Stauffacher, 1988).

In farmed rabbits the doe's parity, which improves from the first to the third litter (Canali *et al.*, 1991) may affect nest quality, female aggression, the whole maternal care and milk yield (Ross *et al.*, 1956, Denenberg *et al.*, 1958, Lukefar *et al.*, 1981, Canali *et al.*, 1991, Gonzalez-Mariscal *et al.*, 1998) as well kits' reactivity and growth rate. In a study carried out by Verga *et al.* (1986) the kits born to multiparous does showed higher exploration activity in the open-field test compared to the ones born to primiparousdoes. Moreover, the latter seem to develop more slowly than kits from multiparous does.

3. Environmental factors

The behaviour of kits may be affected by many environmental variables, mainly the type of nest, which may allow free or controlled access to the doe, as well the material given the doe to build the nest (Verga *et al.*, 1987). Canali *et al.* (1991) found that free-nursed kits show higher freezing times than controlled-nursed kits in the same test. Controlled nursing also seems to induce a higher degree of relaxation in the kits, which is also reflected in a higher growth rate. In a farm, the nest may always be open, thus allowing the mother free access, or closed, thus limiting the nursing period, generally to once a day for a few minutes. According to Coureaud *et al.* (2000) limited access to the nest results in a more than twofold decrease in mortality of primiparous doe kits, although other authors found no difference in kit survival rate due to free or limited access to the nest by the doe (Castellò *et al.*, 1984, Pizzi and Crimella, 1984). A permanently open nest box may cause the kits to leave the nest earlier than those kept in a closed nest box (Baumann *et al.*, 2005). Moreover, a closed nest box with access for the doe limited to once a day may reduce kit mortality rate (Kersten *et al.*, 1993) till weaning (Verga *et al.*, 1978, 1987), and may avoid the stress due to disturbance by the doe (Arveux, 1994).

The quality of the whole nest is another important factor for the survival and growth of kits (Zarrow *et al.* 1963, Lebas, 1974, Mohamed and Szendrő, 1992), and it improves over the first three litters (Ross *et al.*, 1956, Canali *et al.*, 1991). Nest quality is related to the type of material that the doe is given some days before parturition (Verga *et al.*, 1983, Battaglini *et al.*, 1986, Verga *et al.*, 1987).

Another factor that may greatly affect kit behaviour during the first period of their lives is being handled by familiar people, as this lowers their stress reaction due to the 'fear' of humans after weaning (Marai and Rashwan, 2004).

Kits are highly sensitive to handling in the first period of life (Bilkò and Altbacker, 2000). Early stimulation seems to affect the development of their species recognition (Pongracz and Altbacker, 1999), probably based on endogenous factors coinciding with pre-nursing arousal (Allingham *et al.*, 1998). Thus, effective handling seems to be associated with the feeding of kits, and early associative learning of odours may be reinforced with feeding (Brake, 1981). Verga and Zingarelli (2001) and Luzi *et al.* (2002) found that handled kits show less fear reaction in the open-field test and in the tonic immobility behavioural tests, aimed at evaluating fear both towards a new environment and towards human beings. The effects of handling will be dealt in detail in the subchapter on growing rabbits.

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2.5. Behaviour of breeding does in cages

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1. Introduction

Studies on the behaviour of wild rabbits have provided information about the frequency and patterns of their main activities, social organisation and maternal care. Domestic rabbits show similar behaviour, but with changes related to the housing conditions (e.g. light regime, cage size and enrichment). Infrared video observations during a period of 2088 hours in wild and domestic rabbits kept in groups in two enclosures with free access to two nest boxes and other hiding-places, have shown that both wild and domestic rabbits spend a considerable part of their time (54.9 % in wild and 30.6 % in domestic rabbits) in pairs or in groups of three and were in voluntary body contact between 65.3 % (domestic rabbits) and 80.4 % (wild rabbits) of the time. While lying together in a group, both wild and domestic rabbits used only a very small nest box space: 0.08 to 0.125 m^2/animal in wild and 0.14 m^2/animal in domestic rabbits (Selzer and Hoy, 2003). Adult domestic rabbits spend much more time than wild rabbits resting outside the nest box.

Under commercial farm conditions, nests are provided on day 28 of pregnancy, litters are distributed equally among does after parturition, subsequent matings are often performed on day ten post partum and litters are weaned at 28 - 35 days of age. Does easily adopt other kits. As a result, under controlled lactation schemes when does are removed from the litter, other does can easily take their place.

Parturition is expected to change nocturnal behaviour, interfere with some activities and stimulate others, for example nesting behaviour. Does pull out some of their own fur to prepare the nest a few hours prior to partum regardless of light conditions or other circumstances. The doe scratches the floor violently in the hours before parturition for up to 600 seconds per hour if she has no possibility of digging out a hole. She prepares a rough irregular circle with the available material. Some kits may be left out of the nest and will be less likely to survive.

2. Activity pattern

Resting is reduced before parturition and consequentely other activities are increased. The time spent in the nest, apart from parturition, can reach 12 % of total time. Grooming and chewing are linked to the partum surroundings and are increased after parturition. On day one (the day after parturition), the doe seems to compensate for the activities not carried out on the day of parturition (drinking, eating and grooming). On the other hand, less time is spent in the nest nursing the litter. Because the doe is obviously more nervous on day one after kindling, chewing is more frequent and lasts longer and there are more visits to the nest, which do not correspond to the time spent inside the nest. The resting periods are more frequent but shorter on the day after parturition and the next day (143 and 81 seconds/resting) than on days 10 and 28 after kindling (236 and 260 seconds/resting)(Table 1) (Fernandez *et al.*, 2005a, b, Lopez *et al.*, 2002).The results of behavioural observations suggest a trend towards routine behaviour during lactation. Higher negative correlations between the time spent resting and other activities during lactation and the lower coefficients of variation for the activities in lactation compared to those in parturition indicate a change in the species-specific behaviour.

Table 1. Frequency (number of events per hour) of some activities during the day of parturition (P) and during the days 1, 10 and 28 of lactation.

	Drinking	Eating	Caecotrophy	Nest[1]	Grooming	Chewing	Neighbour[2]	Resting
Day P	1.6	2.1	0.9	4.1	9.0	7.1	0.6	13.9
Day 1	4.8	4.2	1.7	4.9	14.8	14.5	2.7	22.8
Day 10	3.5	2.4	0.9	1.3	6.6	4.4	0.4	8.9
Day 28	1.5	2.9	0.7	0.2	6.5	2.5	0.2	9.3

[1]Doe visits or inspects the nest;
[2]Doe stares at her neighbours, tries to touch or attack them

Feeding activity is low on the day of kindling and on the previous days. The time spent eating and drinking is minimal on day of parturition and increases afterwards, following a curvilinear response that resembles the well known curves of food intake or milk yield during lactation. The values published regarding feeding in non-lactating adult animals are highly variable.

Most studies indicate that feed intake occurs mainly at night in wild rabbits. As stated by Vastrade (1985), the results vary more in domestic rabbits because there is a tendency to also use the daytime for feed intake behaviour. Hoy *et al.* (2000) did not detect differences between day and night in feeding of lactating does housed in cages. The results of Fernandez *et al.* (2005a, b) reflect a nocturnal preference, but the feed intake also occurs during the day, especially in the last three hours of the light period, as also reported by Reyne *et al.* (1978). On the day of parturition, does seem to prefer feeding in the dark. It was observed that rabbit does clean the litter after parturition and then they eat and drink.

The frequency and time used for caecotrophy is affected by time and other factors, with a maximum at midday and in the afternoon, as observed by Jilge (1974). Like other activities, the frequency and time for caecotrophy were more than twice as long on day one after kindling compared with the following days.

Grooming takes place at night and represents about 12 % to 20 % of the total time budget, as found by Gunn and Morton (1995), Hansen and Berthelsen (1999) and Fernandez *et al.* (2005a, b). In semi-free colonies, grooming time is much shorter, which suggests a decrease proportional to the increase in social and exploratory behaviour. Some works on wild rabbits have shown that grooming takes place in the early hours of the morning, but according to others, the grooming behaviour of farm rabbits is distributed throughout the whole day (Vastrade, 1985, Gunn and Morton, 1995; Krohn *et al.*, 1999). The high values on day one correspond to the need to clean themselves after parturition. With the increase in the duration of lactation there is a corresponding decrease in grooming activity.

Rabbit does spend about 4% of 24 hours in chewing. The chewing activity increases in the first hours of light and darkness (after dusk or dawn) and is almost three times higher on day one after kindling than on the following days.

Rabbit does are gnawing approximately 5% of the total time budget (Hansen and Berthelsen, 1999, Fernandez *et al.*, 2005a, b). Gunn and Morton (1995) found that bar biting takes up 11% of the total time budget, but they also included fur biting in this behavioural pattern.

Non-lactating does at a late stage of pregnancy rest more than 80% of the time in 24 hours, probably due to their increased weight. The frequency of grooming behaviour in this period is very low (less than 7% - López *et al.*, 2002). On day 28 of lactation, does groom their kits occasionally. The presence of kits in the nest affects the activity of the doe. The increase of doe activities in the morning and at dusk may force kits to enter the nest more frequently.

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2.6. Behaviour of growing rabbits

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1. Introduction

Kits may be completely independent of the mother around the end of the fourth week of age, although the weaning time and process are related to whether the doe is pregnant again or not. This also affects the kits' solid food ingestive behaviour as well as drinking, according to when the mother starts to wean them (Hudson and Altbacker, 1994, Hudson *et al.*, 1996). Thus, weaning at six or even eight weeks may be late both for kits and doe (Hudson *et al.* , 2000).

After weaning the rabbits try to join the social structure of the colony (Kaetzke and von Holst, 1997) and unrelated adult rabbits may show aggressive behaviour (Myers and Poole, 1961) and even infanticide (Kunkele, 1992) towards the young. Adult does are aggressive towards younger rabbits at the end of the breeding season (Denenberg *et al.*, 1969 in Hafez).

Many factors related to housing and management may affect the behaviour of growing rabbits. Being social animals, keeping them in a single cage may lead to stress caused by social deprivation, which may interfere with the development of normal adult behaviour (Marai and Rashwan, 2004). To meet the rabbits' requirements, mainly from the ethological viewpoint, group rearing in pens with straw has been considered as a possible alternative to cages, but problems may arise due to aggressive behaviour. The risk of infestation (i.e. coccidiosis) also has to be of course very strictly controlled. Chu *et al.* (2004) found higher abnormal behaviour (digging, floor chewing, bar biting) in laboratory rabbits housed individually compared to those housed two per cage. The same authors found that, although aggressive behaviour between pair-mates was not a problem, one pair had to be separated due to the injuries from persistent aggression. However they conclude that pair housing may be considered an alternative to single housing for these animals.

2. Aggressive behaviour

Aggressive behaviour may be seen in grouped rabbits, and may be a problem in growing rabbits, because it increases with increasing age, and is at its highest at 80 days of age. These behaviours and the injuries caused by aggressive behaviour may be due to the accelerated sexual development (according to strain, housing and presence of adult females) and to the difficulty in establishing a stable social hierarchy in large groups.

Bigler and Oester (1996) studied 55 groups of fattening rabbits of different group sizes: male, female and mixed-sex groups, reared in pens without or with partial bedding. Other groups were observed in fattening cages, in floor pens or in wooden cages with bedding. The number of animals/group was < 10, 10 - 15, 16 - 30 and >= 40 subjects, while the density was between 3.4 and 6.0, 2.1 and 6.1, 2.1 and 7.3, 7.2 and 8.1 animals/m^2. Both the number and severity of injuries and the aggressive behaviour recorded from 60 to 80 days of age were higher in larger groups, with 16 - 30 and >= 40 subjects. According to the same authors, the levels of injuries and aggressions recorded in their study (from 18 % to 56 % and 64 % in the largest groups) are too high. However, they consider that other causes of aggressive behaviour may be related to "individuality, stocking density, housing systems with different shelters and possibilities for avoiding each other, lighting, etc." Moreover, in larger groups

the hierarchy may be disturbed because of the low control of the environment in a very stressful situation, from the spatial and social viewpoint.

Possible solutions to these problems of aggression have to be studied, for example slaughtering at an earlier age (11 weeks), or the optimum enrichment of the pen or the delay of sexual maturity. Gallazzi (1985) did not observe fights among rabbits before 70 days of age. Similar results have been obtained by Ferrante *et al.* (1992), on rabbits kept in pens at 850 cm^2/head. Heil (1997) found that the frequency and occurrence of biting and the age at its first occurrence vary according to strain; however this behaviour occurs very seldom before the age of 12 weeks.

Mirabito *et al.* (1999) found that group size in cages affects behaviour: 6-grouped rabbits show more active behaviour (locomotion, exploration and eating) than rabbits reared two per cage, at 64 days of age. However this could also be due to the more restricted possibility of movement in the double cage compared to the collective one.

Aggressive behaviour in male rabbits and the severity of injuries do not seem to be affected by lighting regime, as shown by Bigler and Oester (1997), who investigated aggressive behaviour in 16 groups of growing male rabbits reared with light intensities of 5, 15, 30 and 45 lux and under two different lighting schedules (8 L : 16 D and 16 L : 8 D).

3. Preference behaviours

The preference of growing rabbits for different housing systems has also been studied. A big problem for growing rabbits, particularly at the practical farm level, is the quantity and quality of available space and the possibility for rabbits to show "normal" locomotory behaviour (Stauffacher, 1992) and development (Drescher, 1992). According to Lehman (1991) in the rabbits reared two per cage, the ability to perform hopping as well as bone integrity were impaired.

Bessei and Rivaletti (1997) used operant conditioning (pressing a bar) to verify the preferences and motivation of weaned rabbits to gain feed and to choose the amount of available space: from 545 to 3150 cm^2. The animals learned quickly to open a feeder through bar pressing, although they found it easier in a reduced space than in a larger one. Thus the rabbits worked actively to reduce the floor space. However the frequency of bar pressing to reduce floor space was lower than that to increase the floor space. The author concludes that the preferred space may be somewhere in between the two extremes of the test situations.

Matics *et al.* (2004) recorded the choices of different groups of rabbits with different group sizes (18 to 30 and 8 to 24) and space allowance (12 to 20 rabbits/m^2 and 5.3 to 16 rabbits/m^2) from weaning (at three weeks) until ten weeks of age. They used a free choice design with cages of different sizes (500 x 300 - 600 - 900 - 1200 mm) with swing doors between them. Rabbits preferred one of the smallest cages, with a space allowance of 60 - 70 rabbits/m^2 and only a few of them chose the largest cage. After 5 - 6 weeks of age they began to spread into all of the cages, however the smallest cages received a significantly higher preference until the end of the study period.

Princz *et al.* (2005) observed the preference of young growing rabbits housed in cage-blocks of 2 m^2 divided into 4 cages varying in heights of 20, 30, 40 cm and an open-top. Fewest rabbits (less than 17 %) were observed in the open top, and rabbits chose the higher cages when they were active and the lower ones when they were resting, regardless of the space allowance (16 or 12 rabbits/m^2).

4. Time budget

Some research has been carried out on the effects of stocking density and number of animals/cage on rabbits' behaviour. The time budget may be affected by the housing system: different activity and resting times have been recorded in group pens *vs*. cages by Podberscek *et al.* (1991), in fact the total activity percentage is respectively 75 and 66 %. Lehman (1987) reports that rabbits in cages show more displacement activities than those kept in pens. Stereotypies have been found only in individually caged rabbits (Podberscek *et al.*, 1991).

Morisse and Maurice (1997) compared caged rabbits in groups of six, seven, eight and nine, at a density respectively of 15.3, 17.8, 20.4 and 23 subjects/m^2, at six and ten weeks of age. They found that during this period rabbits spend 60 % of their time resting, 10 - 15 % time feeding and 25 – 30 % displaying other activities, without any difference among the treatments, thus confirming the results of other authors who found that rabbits are able to have many feed intakes per day and don't eat at fixed hours (Prud'hon *et al.*, 1972). Sexual behaviour was not observed, as shown also by Lehman (1991), who found that in semi-natural conditions male rabbits do not display this behaviour before 70 days of age. No real stereotypies were observed in the groups, and comfort behaviour (mainly self-grooming) was prevalent at six weeks of age, but without differences among the treatments. Antagonistic behaviour was observed at ten weeks, mainly at the lowest density, but it was difficult to distinguish between true aggression and playing behaviour. Reduced social and locomotory behaviour were observed at ten weeks beyond 6 rabbits/cage (or 15.3 rabbits m^2), and this may be due to the lack of space. On the other hand, comfort and investigative behaviour tended to increase beyond 6 rabbits/cage, suggesting redirected care towards themselves and the environment (cage and equipment). The results

indicate that six rabbits/cage, equivalent to 38 - 40 kg/m^2, may be considered the threshold for the compatible expression of behaviours in caged rabbits. These observations are in agreement with the results of Maertens and De Groote (1984).

Verga *et al.* (2004a, b) studied the behaviour and performance of growing rabbits reared at 2, 3 or 4 per cage (density: 1045, 697 and 522 cm^2 respectively), through time-lapse video-recording at the beginning and at the end of the fattening period (35 and 75 days of age). No differences were found in daily weight gain. The time budget in the two periods is different: after weaning rabbits show mainly alert, movement and eating, while at the end of the growing period they show more exploration, smelling of the environment, as well as social behaviour directed towards the other rabbits. Rabbits at the lower density show less resting behaviour and a higher variety in behaviour compared to the rabbits in the other two treatments.

The time budget of rabbits kept in pairs or in groups of six, during the light period, is different (Mirabito *et al.*, 1999). In the smaller groups rabbits rested more only during the last week of the growing period. The frequencies of locomotion, exploration and social behaviour were higher in groups of six rabbits during the first and last week of the growing period. Also Martrenchar *et al.* (2001) observed the effects of increasing group size (6 *vs* 24 rabbits per group) on rabbit behaviour at six and nine weeks of ages. Rabbits in groups of six at nine weeks of age spent less time resting, while the time spent eating and interacting socially increased. Locomotory behaviours did not change according to the type of housing, but at six weeks the number of multiple hops were lower in cages than in pens, and at nine weeks of age single hops were performed more in the cages. Abnormal behaviour was shown independently of the housing system.

Penned rabbits in group of 100 animals compared to rabbits reared two per cage, at six and ten weeks of age, during the light period, showed higher frequencies of comfort, social and locomotory behaviours and a lower level of resting and feeding behaviour (Dal Bosco *et al.*, 2002).

Postollec *et al.* (2003) did not find significant differences in the average time-budget during the whole growing period in rabbits kept in groups of 6, 10 (with platform) and 60 rabbits. Penned rabbits showed in 51 % of the observations running and hopping, while in cages with six and ten animals this behaviour occurred in 30 % of the sequences. This behaviour was significantly different from the groups of 60 rabbits.

In another research Verga *et al.* (1994) found that rabbits reared in floor pens at a lower density (850 *vs*. 600 cm^2/animal) appear less stressed in open fields than those at a higher density.

The preference for floor type was also studied in rabbits, from weaning (at 21 days) until ten weeks of age, using a choice test, comparing different floor types. The plastic-mesh floor was preferred in the first period after weaning, while, with increasing age, plastic-mesh, wire-mesh and plastic-slat were equally preferred (Matics *et al.* , 2003). Also Morisse *et al.* (1999) found that grower rabbits show a weak preference for straw on the floor. In fact rabbits spent 89 % of time at seven weeks of age and 77 % of time at ten weeks of age, especially when lying (96 % at seven weeks and 84 % at ten weeks) on wire floor compared to a straw deep litter. This choice may be due to the attraction towards the cleanliness and dryness of the wire compared to the littered floor. The whole time budget is not affected by the floor type: resting 60 %, grooming 19 % and feeding 19 – 20 %. Also Orova *et al.* (2004) found that the growing rabbits spent over 80 % of their time on the wire mesh floor, independently of space allowance (12 or 16 rabbits/m²).

The preference for litter may depend on the environmental temperature (Bessei *et al.* 2001): the rabbits could prefer littered floor when temperature is below 20 °C while wire mesh floor when it is above 20 °C. Also the humidity of the litter could shift their preference towards wire mesh.

Some research has also been carried out on alternative floors to wire mesh. No effect was observed of floor type on behaviour (time-budget video recorded during 24 h at 57 and 68 days of age) and bone integrity (tibia and femur dimensions and resistance to fracture) between slatted floor (galvanised steel bars of 2 x 2 cm section and 1.5 cm span) and wire-mesh floor in cages of eight growing rabbits, at two space allowances (12.1 and 16.0 rabbits/m^2) (Trocino *et al.* , 2004).

5. Behavioural tests

Adaptation level of grower rabbits to the housing and management conditions may be evaluated through behavioural tests such as ‘open-field’, ‘tonic immobility’ and 'emergence' test. These tests aim at evaluating the effect of different husbandry systems on the animals’ reactivity, measuring their fear reaction in a new environment or towards humans (Gray, 1991, Erhard and Mendl, 1999). Rabbits’ reactions may be affected by the housing system and management. Better adaptive behaviour reactions in 'open-field' tests have been shown by Ferrante *et al.* (1992) in group pen-reared rabbits compared to those reared in cages, although production did not differ. Verga *et al.* (1994) found different reactivity in the open-field test in rabbits reared at two densities: 850 cm^2/head and 600 cm^2/head. Rabbits at the highest density show lower production and a quicker and more passive (freezing) stress reaction. On the other hand, Xiccato *et al.* (1999) observed no differences in the open-field reactivity in rabbits reared at two densities: 12 *vs.* 16 rabbits/cage.

The results of the open-field test have to be considered from the adaptive and the motivation viewpoints. In fact the adaptive reaction may be, on one hand, the flight reaction, which may represent an attempt to escape from danger (Kilgour, 1975); on the other hand, freezing may be the best way to avoid predators adopting a mimic strategy.

Emergence test and tonic immobility tests have also been performed in order to verify the effects of weaning time on the rabbits' reactions. A trend towards higher emergence times was found in rabbits weaned at 32 days compared to those weaned at 24 days of age. In the tonic immobility test a trend to higher immobility times was found in rabbits weaned at 24 days of age. The differences however were not statistically significant (Verga *et al.*, 2004a). Analogous results were found in rabbits weaned at 21 and 25 days: all the tested subjects showed high locomotory behaviour (number of squares entered), as well as investigative behaviour, in the open-field test. Only freezing times were higher (but not statistically) in rabbits weaned at 21 days of age compared to the others, but high individual variability was also found (Verga *et al.*, 2004a).

The reactivity in the behavioural test may be affected by genetic predisposition also. Two lines of rabbits with high or low reactivity (Open Field Score = OFS) were selected (Daniewski and Jiezierski, 2003), resulting in eight generations of divergent selection with statistically different activity in open-field. OFS is defined by the authors as the number of rectangles in the Open field (OF) entered with both front legs. Estimated heritability for the Low OFS lines in 0 - 3 and 0 - 8 generations were respectively 0.46 and 0.44, while in the High OFS line h^2 was 0.23 in 0 - 3 generations. Males and females did not differ significantly. The divergent selection may correspond to different and consistent coping styles: active copers may show an active response to aversive situations, whereas passive copers may show immobility and withdrawal in the same situation (Wechsler, 1995).

6. Handling

An environmental stressor acting on growing rabbits' welfare is the fear towards humans, who may be perceived by them as predators (Suarez and Gallup, 1982; Price, 1984). This may be reduced by handling the kits during the first period of life (Pongracz and Altbacker, 2003, Marai and Rashwan, 2004, Metz 1983, Kersten *et al.*, 1989). It has been suggested that handling reduces fear towards humans through a learning process of habituation, rather than depressing the general fearfulness (Jones and Faure, 1981, Hemsworth *et al.*, 1986). On the other hand, according to Kersten (1986) rabbits' general emotional fearfulness is lowered due to handling by humans. Some basic research has been carried out on laboratory rabbits in order to verify the effects of handling on physiological variables and on mortality rate. From the physiological viewpoint, Nerem *et al.* (1980) found that handled rabbits fed a diet with 2 % cholesterol (to induce arteriosclerosis) had a 60 % reduction in the occurrence of aortic lesions compared to the non handled ones. Also Duperray (1996) found that, in rabbits repeatedly handled before weaning, at 17 - 20 days of age compared to the non handled ones, the average post-weaning mortality was 3.5 and 6.2 % respectively.

A reduction in fearfulness towards humans has also been shown by Podberscek *et al.* (1991) who studied post-weaned laboratory rabbits reared in pens with deep litter and other rabbits reared in cages. The animals were repeatedly handled by a familiar and subsequently by an unfamiliar person. The rabbits' reactions were classified as 'non fearful' or 'fearful' according to their behaviour reaction: in the first case they accepted being picked up without struggling, they show resting, standing, eating, come and hop forward and exploratory behaviour; in the second they hop away, struggle when handled and try to escape, stamp hind feet, hop back and back away. A significant reduction in fearfulness towards the handlers was shown both by penned and by the caged rabbits during the experimental period, thus indicating that they can learn from experience due to a habituation process. The authors conclude that the implementation of handling and approach programmes could reduce the fear reactions of animals.

Other authors found that handling affects rabbits' fear reaction if it is applied during a sensitive period in the 1st week post partum and near the time of nursing. In fact a sensitive period for early odour learning during suckling has been found in rabbits (Kindermann *et al.*, 1994). Handled rabbits show higher exploration reactivity and seem to be less fearful in the open-field test compared to the non handled ones (Denenberg *et al.*, 1977), thus indicating better coping in a stressful situation. Also Verga *et al.* (2004a, b) found that handling in the first period of life, together with controlled nursing, significantly affects rabbit's reactivity in behavioural tests. Handled rabbits (with controlled nursing) showed after weaning, compared to the ones handled but free nursed or non handled, both controlled and free nursed, higher locomotory activity in open-field (92.1 *vs*. 47.4, 50.5 and 48.6 sec, P = 0.0001) and a higher number of escape attempts (5.53 *vs*. 2.12, 3.02 and 2.09 sec, P = 0.0008). On the other hand, they showed less investigative behaviour. Very low freezing times were observed, thus indicating a reduced fear response in all the subjects.

According to Pongracz and Altbacker (1999), handling by humans around nursing time significantly affects the rabbits' subsequent behaviour. In fact, handled rabbits readily approach

a human hand when tested at weaning, while the rabbits handled 6, 12 or 18 hours after nursing avoid it. The same rabbits when adults show less fear reaction in the open-field test, being more active than the non-handled ones. The well defined sensitive period for successful handling starts 0.25 hr before and ends 0.5 hr after nursing during the first week of age.

The reduced fear is long lasting and specific to the handler species (Pongracz *et al.*, 2001). Bilko and Altbacker (2000) found that handled does have successively higher breeding performance. In fact females handled in infancy showed higher conception rate (86.43 % *vs*. 54.80 %, $P < 0.05$) than the non handled ones. Also the duration of pregnancy was significantly lower (30.63 days *vs*. 31.71 days, $P < 0.05$), although the litter size were the same in the two groups.

Handling seems to have the same effect in domestic as in wild rabbits (Bilkò and Altbacker, 2000), in fact handled rabbits readily and repeatedly approached the test person at weaning during an approach test both in a strain of domestic rabbits and in the wild ones.

Also Jiezierski and Konecka (1996) found that early handling seems most effective in reducing emotionality. They handled kits twice per day from the 10th day of age till 30 weeks for ten minutes each day, taking them outside the cage. They distinguished, according to the rabbits' emotional reactions (freezing *vs*. non-freezing), two categories of animals: the 'timid' and the 'bold'. At 30 weeks of age they found a lower mortality rate in the handled than in the control rabbits (17.5 % *vs*. 31.9 %, $P = 0.055$). Moreover, the handled rabbits were heavier than the controls. The same rabbits were also classified as 69 % 'bold' and 31 % as 'timid', whereas the non handled rabbits were classified as 37 % 'bold' and 63 % as 'timid'. However the effect of genetic strain needs further research.

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2.7. Group housing of breeding does

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1. Group-housing: beneficial for welfare?

1.1. Introduction

An alternative and innovative way of housing breeding does is based on group- or colony-housing. Group-housing facilitates social contact between does, allows more total space and variation, and permits the expression of natural reproductive and maternal behaviour (Bigler and Oester 2003; Bigler 2004; Ruis and Coenen, 2002b; 2004 a, b; Stauffacher 1992). It is advisable to house domestic rabbits in groups, as they still have a need for social interaction, and many analogies exist between the social behaviour of wild and domestic rabbits (Hoy and Selzer, 2002; Selzer, 2000; Selzer and Hoy, 2003; Selzer *et al.* 2004). Greater total space makes a division into functional areas possible (e.g. for resting, a separate area for the young).

In 2001, the Animal Sciences Group, based in Lelystad in The Netherlands, started a series of experiments on the feasibility of group-housing for breeding does (Ruis and Coenen, 2004b), as a result of the growing public concern for the welfare of rabbits kept under commercial farming conditions. Before the first experiment, a thorough study of previous experiences with group-housing was carried out and put together in a report (Ruis and Kiezebrink, 2001). Although the advantages from the welfare perspective may be clear, as decribed above, group-housing leads to major changes in management and housing, and is associated with specific new (welfare) problems.

1.2. Welfare issues in group-housing systems

The most important welfare issues in group-housing systems are:

The free entrance of does to nestboxes of other does may cause high mortality in young rabbits. This may be due to the competition for nesting places, or is caused by accidental crushing of young kits by alien does. Stauffacher (1992) reported that does seemed to have a clear preference for certain nesting sites and that competition could arise if two does were at the same stage of lactation. Recently, Mirabito (2003, 2005 – personal information) even observed that 32.5 % of births took place in a box where a doe had already given birth and, in more than 6 % of cases three does gave birth in the same box. Does that give birth in the same nest seem to tend to be aggresive and try to kill alien kits, particularly when they are born at different times.

Aggression may prevail in groups of does (Bigler and Oester 2003; Bigler 2004; Schuh *et al.* 2003; Stauffacher, 1992), and as a consequence this may negatively affect productivity. Aggression is principally triggered when previously unfamiliar does are put together, when new does are introduced to the group associated with sexual behaviour and pregnancy, and by competition for nesting places.

Group-housing during the breeding period probably also requires group-housing during the rearing period. When does are kept individually prior to group-housing this may result in aggression, e.g. due to the lack of social learning in how to interact with conspecifics.

The system requires especially high standards of hygiene, because of the close contact between animals. Health control may be more difficult.

1.3. Other important implications of group-housing

The system is by its complexity labour-intensive: monitoring of the breeders and litters, catching and cleaning is more difficult.

The difficulty of identifying young rabbits makes selection of breeding does more difficult.

Production costs in group-housing systems are expected to be higher than in regular individual housing systems.

2. Development of the IENR technique

As mentioned above, the free entrance of does to nestboxes of other does is one of the main problems in group-housing, causing high mortality in young rabbits (Mirabito 2003, 2005 – personal information). It was expected that this problem could be solved by the use of an individual electronic nestbox recognition (IENR) system, allowing only the doe to have access to her own nestbox. In The Netherlands, it was hypothesized that the IENR system should be the basic component for a group-housing system, and therefore had to be developed first (Ruis and Kiezebrink 2001).

The first design of an IENR system had the following characteristics:

- A chip was attached to the ear of the doe (Fig. 1).
- Each chip opens only one door in a tunnel-like link to a nesting box. Each square tunnel had a length of 35 cm and had inner dimensions of 16 x 16 cm (Fig.2).
- The door is opened by the chip when a doe enters the tunnel from the pen, but the door can be opened freely by the doe and kits when entering the tunnel from the nesting box.

Figure 1. *Chip in ear of doe.*

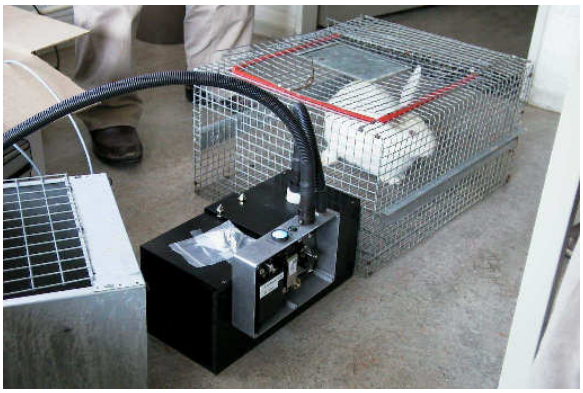

Figure 2. *Tunnel-like link to nesting box, including a door to be operated by a chip.*

3. Development of a group-housing system

Between 2001 and 2005 several experiments were performed in The Netherlands, and a prototype of a group-housing system was developed stepwise. The feasibility of the system under commercial conditions was also investigated. In all experiments, New Zealand White rabbits were used.

3.1. Pair-housing

It is known that pair-housing of breeding does may lead to serious aggression problems, especially when the animals are close to giving birth (Reichel, 1995). Whereas in groups of more than two does, animals may spend a considerable amount of time together (≥ 30 % (Mirabito, 2003; 2005 – personal information) to 50 % of time (Stauffacher, 1992), this was only 0.8 % of time with paired does, kept in paired cages (Mirabito, 2003, 2005 – personal information). In the latter study, in two out of nine pairs, there was a situation of mutual exclusion, in which each female remained in its own cage, and the investigators had to cull some of the pairs over the course of the experiment.

3.2. Introduction of the IENR technique and effect of social bonding

The above described findings indicate that housing does in pairs is not promising from a welfare and reproductive perspective. However, it is not known whether the negative results with paired does also occur when there is no competition for nesting boxes, by introducing the IENR technique. Moreover, it may be expected that aggression is influenced by familiarity or bonding between two does. Therefore, an experiment was performed with five pairs of littermates and five pairs of non-littermates (Ruis and Coenen, 2002a). Importantly, the IENR technique was accepted without problems by the does. However, aggression was highly influenced by familiarity between does. In three pairs of non-littermates, animals either died or had to be removed due to high aggression. In addition, does

of non-littermate pairs showed a higher frequency of external wounds caused by fighting. Reproduction performance was not affected by familiarity, as litter size and numbers of stillborn young did not differ. The degree of familiarity did not affect body growth either.

3.3. A modified group-housing system

The next step was to design a suitable prototype of a group-housing system, and for this purpose the basic elements of the Stauffacher system were used. Stauffacher (1992) proposed a housing system with enclosures of 2 m by 4.5 m designed to accommodate one male and four to five females. An enclosure comprised different areas, one for feeding (containing food and drinking troughs and straw racks), one for breeding (with litter on the floor and nest boxes), and in between an enclosure also containing various enrichment structures (shelters, platforms, etc).

3.4. Characteristics of the Dutch system

The modified system had the following characteristics (Fig. 3 and 4):

- The IENR system, first tested in pair-housing, was used to give a doe unique access to her own nest.
- A group consisted of eight does, one buck, and offspring until weaning. Does were placed together at 17 - 18 weeks of age. A buck was introduced 5 - 7 days later.
- Total floor dimensions of the system were 2.5 x 1.8 m.
- Nesting boxes were elevated, in order to create a resting area underneath the nesting area. The elevated floors to reach the nesting places were made of solid wood.
- The floor consisted of an artificially slatted floor (Termaat, black). Part of the floor (1 x 1 m) in the resting area was bedded with straw.
- In the feeding area, two pellet feeders were provided, several nipple drinkers and a hay rack.
- A kit area was created only accessible for kits. In this area, kits were able to feed, drink and rest separately from the adult animals.

3.4.1. Low mortality of young rabbits

Mortality of young rabbits was comparable to that in individual housing. This emphasizes the importance of implementation of an individual electronic nestbox recognition system in the group-housing system (Ruis and Coenen, 2002b; 2004b; Table 2.6)

3.4.2. Low aggression

Numbers of skin lesions were used as an indication for aggressiveness. Small and superficial bites were observed around the formation of groups (head and ears: 63 %; body and limbs: 17 %), around births (head and ears: 30 %; body and limbs: 35 %), and around replacement of does (head and ears: 17 %; body and limbs: 13 %), but on average the frequency was rather low and seemed to be the result of functional fighting for establishing and maintaining the social hierarchy. Moderate (average head and ears: 1 %; average body and limbs: 4 %) and severe injuries (average head and ears: 0 %; average body and limbs: 1 %) were rarely observed throughout the experiment (Ruis and Coenen, 2004 a, b). No aggressive behaviour by adults towards kits was observed.

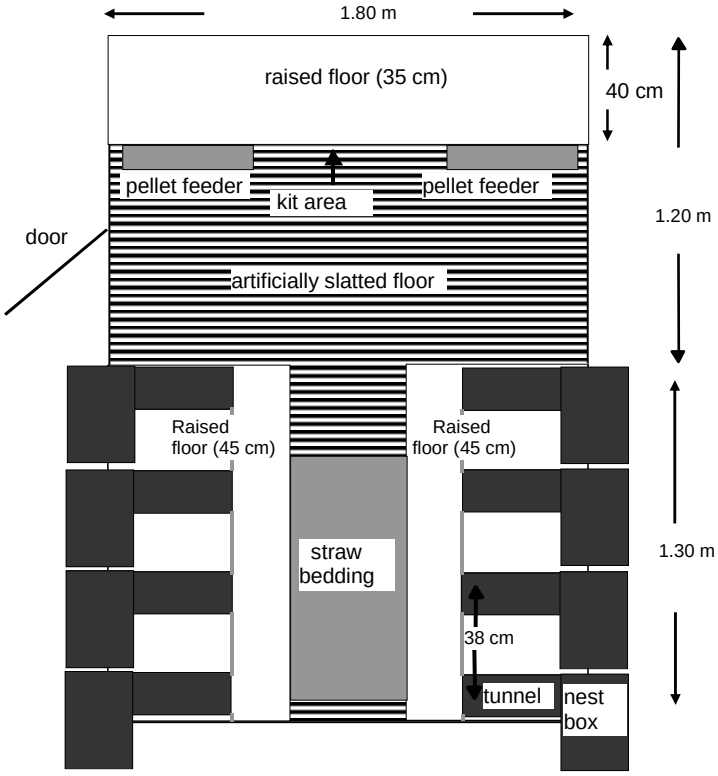

Figure 3. *The first prototype of the modified group-housing system in The Netherlands.*

Figure 4. *Part of the group-housing system: artificial slatted flooring, straw area, raised nesting places with the IENR system.*

3.4.3. Breeding results are comparable to individual housing

Total litter size, the number of kits born alive and culling, did not significantly differ between group and individual housing. In Table 1, results of three groups of does compared to those of 20 individually housed does (cage dimension of 50 x 60 x 30 cm) are presented. The latter animals were inseminated 10 days after giving birth (semi-intensive breeding. In group-housing a buck was present (post-partum breeding). The experiment lasted six months (Ruis and Coenen, 2004b; Ruis *et al.*, 2003b).

Table 1. Breeding results of individually and group-housed does.

	Individual Semi-intensive	Group-housing Post Partum
Total litter size	8.52	9.02
Kits born alive	7.33	8.32
Culling until 14 days	10.6	10.2
Weight of kits at 14 days (g)	257.4	255.7
Culling between 14 days and weaning	1.3	0.8

Results of three groups of does compared to those of 20 individually housed does (cage dimension of 50 x 60 x 30 cm). The latter animals were inseminated 10 days after giving birth (semi-intensive breeding; SI). In group-housing a buck was present (post-partum breeding; PP). The experiment lasted six months (Ruis and Coenen, 2004b; Ruis *et al.*, 2003b).

3.4.4. Low frequency of visits to nests

In contrast to the often observed and undesirably high number of visits to nests in individual housing, the number of visits to nests in the group-housing system was low, which can be attributed to the use of a tunnel-like link to nesting places, necessary for the IENR-technique (Coenen *et al.*, 2002). The number of short visits (less than 60 seconds) was limited to three to seven per day (reducing with age), and daily number of nursings (visits longer than 60 seconds) was between two and three.

3.4.5. Hygiene is not sufficient

The solid floors and the straw area became contaminated by manure and urine. The hygiene of the first prototype therefore did not reach the desired standards, and had to be improved. The part of the floor bedded with straw and the elevated solid floors to reach the nesting places were especially affected by manure (Ruis *et al.*, 2002). In a second experiment, special attention was given to reducing hygiene risks, and therefore an alternative method of providing straw (loose or in rack) and a different structure of the elevated floors were tested (solid or slatted). It was again shown that parts of floor bedded with straw, and solid elevated floors become very dirty (on average 50 % covered with (smears of) droppings (Coenen and Ruis, 2003). The risk of coccidiosis was assessed by counting the numbers of oocysts in the manure. As shown in Table 2, oocysts were always present in group-housing, and could not be found in individual housing after some time (Coenen and Ruis, 2003). It therefore seems that the interaction between animals is a risk factor, in addition to the extent to which animals are in contact with manure.

Table 2. Number of oocysts in manure.

Floring type	After 1 month	After 2 months	After 3 months
Solid elevated floor, straw bedding	+	+/-	+/-
Slatted elevated floor, straw bedding	+	+	+/-
Slatted elevated floor, straw rack	+/-	+/-	+/-
Individual housing, wire of 2.05 mm	+	0	0

Numbers of oocysts in manure: many: +; moderate: +/-; none: 0

Results were obtained in two trials (each three months; only first litter), leading to observations of three groups of does per treatment. In total 20 does were housed individually, on wire floors of thin metal wire (Coenen and Ruis, 2003).

3.4.6. Absence of a buck does not lead to social instability

In the experiment described above (Coenen and Ruis, 2003), it was also tested whether a group of does may also co-exist without the presence of a buck. It is known that a linear hierarchy exists in groups of does, and a male could have a moderating effect on the aggressive interactions between females (Stauffacher, 1992). In the current experiment, the absence of a buck did not lead to social instability and more aggression between does (Ruis *et al.* 2003a). Schuh *et al.* (2003) and Hoy and Schuh (2004, 2005) have shown by analysing the social structure in groups of wild and domestic rabbits kept in enclosures that bucks are not involved in the social interactions between does.

4. Group-housing on Dutch commercial farms

From the year 2003 onwards, the research on group-housing in The Netherlands continued on three commercial farms.

4.1. Characteristics of the system

From the experiences and results of the first experiments, a next prototype of a group-housing system was designed. It had the following important changes:

- The IENR system was modified (second design). The tunnel-like link to the nesting box was transformed into a round plastic pipe. This more resembles the shape of tunnels in the wild, but also decreases costs since the production process is simpler and the material is cheaper.
- The pen is made of a more durable material, i.e. metal instead of wood.
- In the kit area, feed and water were no longer provided, as kits prefer to eat together with the adult animals.
- The following changes are made to improve hygiene, or to study the best way to do so:
- The raised floors consist of artificial slats (MIK, colour green; opening size 10 x 65 mm) replacing the solid wooden floors.
- A hay rack was used for hay and straw. Straw is no longer offered loose on the floor.
- Each pen has a different floor. One pen has a wire flooring (diameter wire: 3 mm), one has a MIK flooring (artificial slats; colour green; opening size 10 x 65 mm), and the third has a Paneltim flooring (artificial slats, orange; opening size 28.1 x 10.9 mm; Fig. 5).

Figure 5. *Group-housing pen with Paneltim flooring. Elevated floor consists of MIK slats.*

4.1.1. The elevated floor is an important structure to facilitate resting and grooming behaviour

The does were often seen below the elevated floors (Table 3), and there they performed much of their resting and grooming behaviour (Rommers *et al.*, 2005b). This confirms the findings in a preference test, in which does could choose between different degrees of 'shelter'. Does prefer a resting place closed on two sides (at one side and above). Such a slightly dark site provides protection and safety on one hand, and an overview for the doe on the other hand (Coenen *et al.*, 2004).Table 3. Use of the pen, and percentage resting/grooming behaviour

4.1.2. Aggression is only occasionally high

Aggressive interactions between does were rarely observed, but in some cases the prevalence of moderate and more severe skin lesions revealed that aggression had become a problem (Rommers *et al.* , 2005b).

Table 3. Use of the pen, and percentage resting/ grooming behaviour.

Location	Presence % animals	Resting/grooming % animals
Total	100	83
Below elevated Floor	66	66
Middle of pen	8	9
At feed trough	12	14
Elevated floor	8	11
Nests	5	-

Average results obtained in the course of two experiments (six months each) on three farms, independent of flooring. On each farm, 24 does were housed in breeding groups (Three groups of eight does)(Rommers *et al.*, 2005b).

4.1.3. Footpad injuries remain a problem

Surprisingly, the number and severity of footpad lesions was high on alternative plastic slatted floorings, as well as on the alternative flooring existing of thick wire with a diameter of 3 mm (all types of floors: between 20-25 % of animals with moderate to severe injuries) (Rommers *et al.*, 2005a). It is hypothesized that the permeability of these floors was too low, leading to more manure on the floor and more moisturizing. This could also produce problems of hygiene, although it was not found to lead to more health-problems.

4.1.4. Breeding results are comparable

In agreement with our previous results (Ruis and Coenen, 2004b; Ruis *et al.*, 2003b), the reproductive performance in group-housing reached the same standards as for the regular individual housing (Table 4). However, a retardation of growth was observed with kits in group-housing, as observed at the age of 14 days (Rommers *et al.*, 2005a). This may be caused by lower milk intake by the kits, possibly related to a lower milk production of the does or to multiple sucklings by alien does in the pen.

5. Conclusions and recommendations

5.1. IENR technique

The successful implementation of group-housing under commercial conditions depends on the presence of an individual electronic nestbox recognition (IENR) system, allowing only the doe to have access to her own nestbox. With such a system, killing of kits by alien does is prevented, and competition for nesting places is eliminated. Other benefits for welfare, very likely related to the presence of an IENR system, are a reduction in aggression between does, and a low frequency of visits to the nests. IENR systems should therefore be further developed to optimize technical operations and be made available to farmers at minimal costs.

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Table 4. Breeding results of individually and group-housed does (Rommers *et al.*, 2005a).

	Individual Semi-intensive	Group-housing Post Partum	P (exp. 1)	P (exp. 2)
Total litter size	9.3	10.1	NSo	NSo
Kits born alive	9.0	9.7	NSo	NSo
Culling until 14 days	6.7	8.7	NSo	P<0.05
Weight kits at 14 days (g)	260	238	P<0.05	P<0.05

Average results obtained in the course of two experiments (six months each) on three farms. On each farm, 24 does were housed in breeding groups (three groups of eight does). In the same room, between 24 and 48 does were individually housed as controls. The latter animals were inseminated ten days after giving birth (semi-intensive breeding; SI). In group-housing a buck was present (post partum breeding; PP).

5.2. Functional areas

The possibility of dividing total space into functional areas is advantageous for welfare. The animals have no restriction in moving around, and are able to adopt an upright position (no ceiling). As a result, specific stereotypies do not occur, and skeletal anomalies are eliminated. In the current Dutch system, four main areas are created:

- Feeding area, containing a food trough, nipple drinker and a hay/straw rack,
- elevated breeding area, consisting of the elevated floors, tunnel with the IENR technique, and nestboxes,
- resting and grooming area, created below the elevated breeding area and
- a kit area, only accessible for kits.

5.3. Group-size and -composition

Although aggression may always occur in groups, and sometimes may even prevail, it seems that group-sizes of five to eight breeding does are feasible for commercial farms. It is not advisable to house breeding does in pairs, as one doe will often dominate the other, leading to serious aggressive encounters, especially around the time of giving birth. Pair-housing thus does not seem not very promising from a welfare perspective, although aggression seems to be at much lower levels in pairs of familiar does (littermates) and may depend on the level of competition for nesting places.

5.4. Hygiene and health

Hygiene is greatly affected by the way in which roughage is provided. Loose material on the floor leads to pollution with manure, and this in undesirable. It is therefore recommended not to provide straw and/or hay as loose material on the floor, but instead to use a rack.

Neither are solid floors recommended, as these also become covered with (smears of) droppings and urine. Floors should be perforated, and from a welfare-perspective preferably not made of thin metal wire. It is therefore important to investigate suitable floorings (for the whole system, including elevated floors). However, alternative floors such as plastic slats are less durable, are more difficult to clean, and have a lower permeability than thin metal wire, and therefore negatively affect hygiene. Although it may be clear that the separation of the does from their own faeces is very important to prevent or to reduce possible infection with endoparasites and the occurrence of infectious diseases, it should also be borne in mind that close contact between does also presents a risk factor for transmission of pathogens.

5.5. Footpad injuries

Floorings for group-housing are still a major concern. Footpad injuries also occur on plastic slatted floors, probably due to a too low permeability

and moisturizing of the floors. Great attention should be given to this aspect of housing.

5.6. Synchronisation of production

The knowledge that the absence of a buck among does does not lead to socially unstable groups, may be of great importance for the feasibility of group-housing and may open the possibility of introducing artificial insemination (AI) into the group-housing system. AI is widespread in commercial production and offers the advantage of synchronisation of production of offspring and better genetic and hygienic control. However, it is not known whether pseudopregnancy will occur more often, thereby impairing performance. It is intended to investigate this topic in future research.

5.7. Lower body weight of young rabbits

The retardation of growth of young rabbits in the group-housing system should be further investigated. Lower weights at a young age may result in lower weights at the age of slaughter or a prolonged period of fattening.

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2.8. Single housing of breeding does

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1. Introduction

In most cases rabbit does are housed individually in 40 - 45 cm wide cages, with a length of 85 - 95 cm and height of 33 - 35 cm, including the nest space. These cages are used for does from some days before kindling till weaning. The minimum size of the nest box is W: 35 - 45 cm, L: 24 - 27 cm, H: 30 - 38 cm. The young and non-pregnant/lactating does are often kept in somewhat smaller cages (W: 30 - 38 cm, L: 40 - 43 cm, H: 33 - 35 cm). Dual-purpose cages are used for breeding and fattening, and kits are reared until slaughter in the cage where they were born.

2. Flooring

The floor of breeding cages is mainly built of wire netting. The diameter of the wire on the bottom is 2.5 - 3 mm and is rectangular in shape (73 x 13 mm). There is an increasing tendency to use plastic footrests. In some cases the bottom of the cage is a slatted plastic floor. According to the observation of Rosell (2003) footpad injuries are a serious problem in intensive production rabbitries with a frequency of 10.4 and 5.7 % among females and males, respectively.

Rommers and Meijerhof (1996) investigated eight different floor types and found that they do not affect the productivity of does, but footpad injuries were closely connected to cage flooring. The percentage of foot injuries increased mainly after the second litter. On the wire floor the incidence and the average score of footpads were higher than on the alternative floors. About 80 % of the animals on wire flooring had severe foot problems after the fourth litter, whereas on the alternative floors this percentage remained below 30 % on average. The results suggest that the smoothness of the floor may be of importance in the prevention of footpad injuries. After a three-year evaluation of animals of five origins, Rossel (2003) registered 9.2 % (range between 0 and 40 %) footpad injuries in 115 rabbitries. The effects of origin and footrest (presence or absence) were significant. Providing footrests can reduce both the prevalence and the seriousness of footpad injuries.

Petersen *et al.* (2000) examined slat distance on the behaviour of does. The width of all slats was 10 mm and the distance between them was 10, 12, 14 and 16 mm. While 21 % of does on the slatted floor with 10 mm slat distance were found to lie in a position parallel to the slats, this percentage was much lower on floors with 16 mm slat distance (7 %). On floors with larger slat widths the does preferred to orientate themselves crosswise on the slats. Schlender-Böbbies (1999) found no significant effect between the studied floor and the degree of footpad injuries. Besides the plastic slatted floors, a wire netting floor with 14 mm distance between wires was tested for young rabbits. The floor type had no significant effect on behavioural patterns of the kits. Considering other aspects, e.g. hygiene and the incidence of pododermatitis as well, only plastic slats with a distance of 14 mm are regarded as acceptable.

To sum up, it can be concluded that the provision of footrests on wire netting floors is recommended to provide a comfortable resting area and to avoid footpad injures.

3. Walls

The walls of breeder cages are mainly built of wire, though sometimes also of solid metal sheets. Solid walls can be advantageous if there are strong

air currents in the building but they prevent any connection between the individually housed does.

Negretti *et al.* (2004) examined the social behaviour of young bucks housed in a set of three contiguous two-floor cages permitting them to be in visual contact each other. The authors observed that the rabbits located in the external cages were preferentially looking towards the nearest one, while the latter, located in the central cage, showed a lack of preferences.

Gacek (2002) examined the effect of visual contact on the reproductive performance of does. The conception rate was the lowest in does without visual contact of the surroundings. In another experiment (Szendrő, 2005 – unpublished data), the conception rate and the litter size were identical in cages with wire netting or solid walls, while the total litter loss was higher in cages with metal sheet walls. This may be connected to the does being frightened, since they are only able to notice a person when he is very near (above) the cage.

From the view point of welfare, wire netting walls allow individually housed rabbit does to have social (visual) contact with their neighbours.

4. Feeders and drinkers

In general, automatic feeders and nipple drinkers are used in commercial rabbit farms, and only limited experimental results are available in this field. The length of feeders used in the current praxis is adequate. The feed consumption of weaned rabbits was not modified when length of the feeders was extended to 45 or 22.5 cm for seven animals (Remois *et al.* , 1999) or 40, 30, 20 or 10 cm for 13 animals till nine weeks (Orova 2005 – unpublished data).

As Maertens and De Groote (1991) pointed out, before weaning dietary requirements for does and kits are different, thus making different feeders necessary. Messager (1993) showed that young rabbits given a pre-weaning pellet from a special feeder allowing access to the kits but not to the doe to the feed from 15 days of age led to a 30 - 50 g weight advantage as compared to the control, while the mortality between three and six weeks was decreased by 5 %. Fortun-Lamothe *et al.* (2000) described an original system for separated feed intake control of dams and their litters in the same cage. The feeder for does was modified to prevent pellet kits ingesting pellets and to prevent the doe from accessing the kits' feeder. This system did not affect milk intake or growth of suckling rabbits.

On farms, it may be expensive to establish a system for separate feeding of does and kits instead of using automatic feeders.

Different drinker types (open type: swimmer bowls or low-pressure bowls, nipple drinker and automatic minidrinkers) were compared to investigate drinking behaviour and to evaluate drinking applicability by Drescher and Hanisch (1995). In this study, the low-pressure bowl drinker was mainly preferred, followed by the nipple drinker. Gábor *et al.* (1988) compared the open type and nipple drinkers and showed that the average water intake increased by 1.5 % and milk production of does increased as well by 9.0 % in rabbits using open type drinkers. Similar results were published by Szendrő (1988).

Maertens and De Groote (1990) examined three types of drinkers (normal and lowered nipple or lick-drinker). Compared to the normal nipple (22 cm above the bottom of the cage), neither the lower nipple drinker (10 cm above the bottom), nor the lowered lick-drinker had a positive effect on litter weight at weaning.

The position of the drinker (near to the wall) is important, allowing easier access by leaning against the wall or other animals (doe or kits).

In spite of improving the performance of does and kits and being easier to drink from, open type drinkers are not recommended because of the higher chance of water contamination and therefore higher risk of diseases. Accordingly, nipple drinkers are more hygienic.

5. Nest box

Usually the width (40 - 48 cm) and height (30 - 35 cm) of the nest box is similar to the size of the doe's cage, while the length is 24 - 27 cm. In most cases the nest space is a part of the doe's cage (built-in), but it can also be located outside. The entry to the nest box should be capable of being closed.

Generally wood shavings are used as litter, though sometimes other materials are applied as well. Two to three days are necessary for the does to prepare the nest for kindling (Matics *et al.*, 2002), therefore the nest box has to be hung up outside or has to be created inside (insert the tray and its front wall) on the 28th day after mating/insemination. If the pre-kindling preparation time is shortened, does may build fragile nests (Matics *et al.*, 2002).

Controlled nursing is a widespread management form, but free nursing is also used. Comparing these two methods, contradictory results have been published. Pizzi and Crimella (1985) found no difference in mortality and in litter weight. In contrast, higher kit mortality was recorded in the controlled nursing group in the experiment of Constantini *et al.* (1986). Arveux (1994) suggested using controlled nursing to reduce kit losses. According to the results of Le Normand *et al.* (1994), the mortality from birth to ten days of age was significantly lower with controlled nursing than with free suckling, though the tendency was reversed afterwards. Conflicting results were published by Szendrő *et al.* (1999), who found that free nursing proved to be better in the first week after parturition but controlled suckling gave lower

mortality between week one and three. The combined method (free nursing during the first week and controlled afterwards) was found to be the best. It seems that the milk production of does (litter weight) was not affected by the nursing method.

Comparing different nest access (free /F/, controlled with closed entrance /S/ or with the nest box placed outside at nursing time /R/) Baumann *et al.* (2005) observed more short nest approaches in group F than in group R, whereas group S performed more medium length approaches than group R and more long approaches than R and F. However, mortality and weaning weight of kits did not differ significantly between the groups.

The effect of nursing methods depends on the parity (Coureaud *et al.*, 1998). In primiparous females, controlled nest access resulted in lower mortality (8.1 %) as compared to free nursing (18 %). In the experiments of Szendrő *et al.* (1999), kit mortality in the group of primiparous does was also significantly lower if controlled nursing was applied (6.4 *vs* 12.2 %), while the opposite results were achived for multiparous does (11.4 *vs* 6.3 %).

A special nursing method was described by Szendrő *et al.* (2000a, b). Two does were housed separately and both of them had controlled access to nurse the kits in the same nest box. Nursing by two does had several advantages. Kits accepted both does as they could suckle both in the morning and in the afternoon or evening. They consumed more milk, the weight gain before and after weaning was higher and kit mortality was lower than in the traditionally nursed group (nursed by one doe).

6. Cage size

Only a few experiments were carried out investigating the effect of cage size on the reproductive performance and behaviour of does. Rommers and Meijerhof (1998) examined rabbit does housed in two different sized (standard: 50 cm W, 60 cm L, 30 cm H; large: 100 cm W, 60 cm L, 30 cm H) or tall cages (50 cm W, 60 cm L, 50 cm H), and the type of cage had no significant effect on fertility. There was a tendency for the litter size at birth and at weaning and the average weaning weight of rabbits in some litters to be significantly higher in large cages than in standard cages. Some of the reproductive traits (litter size and individual weight of kits) were better in tall than in 30 cm high cages. The cage size did not appear to have significant effect on the behaviour or welfare of females.

Mirabito *et al.* (2004 – personal information) compared three different sized cages (3420 cm^2, 4508 cm^2, 5880 cm^2) but, in contrast with the results described above, they did not observe any difference in fertility, litter size, mortality or weight of kits. Time-budgets were also similar in the three cages.

The size of cage has an influence on nursing behaviour. Selzer *et al.* (2004) observed a moderate tendency to decrease nursing activity by increasing cage size.

On the basis of the contradictory results, it is impossible to give a recommendation concerning the size and height of breeding cages.

7. Platform

One of the aims of building a platform in a "two-floor" cage is to increase the floor surface, maintaining unchanged the base area of the cage. The walking surface may be increased by 70 - 80 % (Margarit and Finzi, 2000). The other function of the platform is to keep does away from their kits when they leave the nest-box and want to suckle at any time of the day.

According to the observation of Finzi *et al.* 1996), rabbit does spend about half the time on each of the levels of the cage. When the rabbits were introduced in the upper floor, the time duration spent on the lower level was 24, 51, 74 and 81 % during the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} days after introduction, respectively (Negretti *et al.*, 2004). In another experiment, Margarit and Finzi (2000) showed the effect of first introducing does in the upper or lower level. When does were first introduced in the upper floor their feed intake and water consumption was similar in the upper and the lower levels. When they were first introduced in the lower floor the feed consumption (134 *vs*. 59 g/day) and water consumption (276 or 390 g/day, in the upper and lower floor, respectively) was contradictory between the two levels. Keeping the does for three days on the lower level before opening the passage to the upper one, feed intake (85 *vs*. 173 g/day) and water consumption (175 *vs* 450 g/day) was significantly higher on the lower floor. The variability of the individual behaviour was high in each case.

Cages with a platform may cause hygiene problems if it is solid. Manure can accumulate on it, though if it is of wire netting droppings and urine fall onto the kits, feeders and drinkers. To keep the upper floor clean, to force does to choose the lower level for defecation and urination, Margarit and Finzi (2000) suggest closing the entrance of does to the upper floor for some days. This should be a method of obliging the animals to choose the lower floor to deposit their droppings in order to maintain the upper part clean. However, this theory has not been confirmed by others.

Mirabito *et al.* (1999 – personal information) examined the platform choice. During the light period the lactating does spent 20 to 35 % on the platform with an increasing trend between week two and four. After leaving the nest box, the young began to spend time on the platform as well (5^{th} week: 10 %). The group of non-lactating does spent

27% of their total time on the platform, accordingly, platforms do not appear to be a means for the does to escape from the young and rest unmolested.

In another experiment (Mirabito, 2002), the kits were left with their mothers only for nursing and they were then moved into another cage. Between weeks three and five does in this group spent 12 - 16 % of their time on the platform, whereas in the control group (free nursing during the lactation period) the relevant data were 32 - 42 %. These results show that the presence of kits caused higher occupation of the platform but the presence of a platform did not affect the frequency of nursing attempts.

In the next experiment, Mirabito *et al.* (2004 – personal information) examined the effect of cage size (0.25, 0.30 and 0.44 m^2). The does in the smallest cage spent significantly less time on the platform during lactation than those in the largest one, accordingly, cage size is not a basic element for defining the frequency of platform usage. In the smallest cages does did not jump on the platform to prevent nursing attempts, while in the larger cage the does escaped to the platform or into the plastic tunnel when the kits tried to suckle. The suckling attempts were higher in the furnished cages than in the control ones. The main conclusion of Mirabito *et al.* (2004 – personal information) was that the platform is not an ideal system to reduce the frequency of nursing attempts. In contrast, Selzer (2000) demonstrated that does react to attempts by kits to suck in 89.5 % of all cases by jumping onto the platform. In the unstructured concrete box, the doe has only the possibility of lying down (80.7 %) or running away (13.8 %) as a reaction to attempts of kits to suck. Comparing reproductive performance (conception rate, litter size, mortality, weight of kits and feed consumption), there was no difference between traditional and enriched (double height with platform) cages (Mirabito *et al.*, 2004 – personal information). Further research is necessary to demonstrate the possible effects of the application of a platform in a two-floor cage on behaviour, hygiene, health and performance of does and kits.

8. Conclusion

Summarizing the published results it can be finally concluded that the cages (size, equipment etc.) used in rabbitries are suitable for production and also that they have no harmful effects on welfare. The use of foot-rests on wire-netting floors, and constructing cages with wire netting walls is recommended. However, further experiments are necessary to find the optimal cage size, to find the best nursing method and to avoid the suckling attempts of kits.

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2.9. Environmental enrichment in growing rabbits

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1. Principles and applications

In intensive breeding systems growing rabbits are usually kept in wire-grid cages which represent a very barren environment without any sort of stimuli (Hansen and Berthelsen, 2000). Such an environment prevents rabbits from performing their natural behaviour such as hopping, running, jumping (Lehmann, 1987; Maertens and van Oeckel, 2001), rearing up on the hind legs (Gunn and Morton, 1995), foraging and digging (Podberscek *et al.*, 1991), and precludes their normal exposure to variations of odours, textures and diets (Gunn and Morton, 1995). Deprivation of the full expression of the behavioural repertoire, which, in spite of many years of domestication very similar to the behavioural repertoire of its wild counterpart (Love, 1994, Podberscek *et al.*, 1991, Held *et al.*, 1995), can result in an increased state of stress. Animals become restless and change their behaviour more frequently (Lehmann, 1987, Metz, 1987).

Several kinds of abnormal behaviour such as biting, chewing or licking the bars, feeders, walls and the grid floor, excessive fur pulling, pawing or digging in cage corners, head weaving, etc., appear (Laboratory Animals, 1993). Such abnormal and stereotyped activities are very often a sign of frustration, anxiety or boredom (Laboratory Animals, 1993) and are considered as indicators of mental suffering (Dawkins, 1977, cited by Gunn and Morton, 1995) and reduced welfare (Newberry, 1995).

A most promising method of improving rabbits' living conditions and welfare is to ameliorate husbandry conditions by enriching them (Marashi *et al.*, 2004). Environmental enrichment is defined by Newberry (1995) as any modification in the environment of captive animals that seeks to enhance their physical and psychological well-being by providing stimuli meeting the animals' species-specific need. It should:

- improve the quality of the captive environment so that the animal has a greater choice of activity and some control over its social and spatial environment (Newberry, 1995):
- increase behavioural diversity,
- reduce the frequency of abnormal behaviour,
- increase positive utilization of the environment,
- increase the animal's ability to cope with challenges (Young, 2003, cited by Baumans, 2005).

Environmental enrichment for rabbits may be obtained by provision of social companions (Chu *et al.*, 2004, Love, 1994, Podberscek *et al.*, 1991), modification of rearing system structure by adding places to hide and rest to the cage, such as elevated platforms, closed boxes, alternative floors (Ruis, 2004), additional roughage food objects such as hay (Berthelsen and Hansen, 1999, Lidfors, 1997), grass-cubes, gnawing sticks (Love, 1994) to satisfy the animals need for chewing, and it has even been suggested that music may serve as a form of sensory enrichment (NRC, 1996, cited by Baumans, 2005).

2. Environmental enrichment research in COST Action 848

Certain environmental enrichment was usually provided to animals on the basis of what was intuitively perceived as important for them, was inexpensive and made of locally available materials (Olsson *et al.*, 2003). But nowadays it is becoming increasingly important to evaluate environmental enrichment according to the benefits to animals, by examining the use of and preference for certain enrichment elements, the effect on species-specific behaviour and performance and the effect on physiological parameters (Baumans, 2005). In the frame of COST Action 848 Workgroup 2 "Welfare

Table 1. A summary of studies within COST Action 848 of the effects of environmental enrichment on rabbits' behaviour.

Experiment	Maertens and Van Oeckel (2001)		Jordan *et al.* (2003)	Luzi *et al.* (2003a)	Jordan *et al.* (2004)			Maertens *et al.* (2004)	Princz *et al.* (2005)	Verga *et al.* (2005)
Enrichment	Wooden stick	Straw in a hopper	Wooden stick (Norway spruce)	Wooden stick (Robinia)	Wooden stick (Oak)	Wooden stick (Lime)	Wooden stick (Norway spruce)	Plastic platform + hiding box + wooden stick	Wooden stick	Wooden stick (Robinia)
Housing	Group	Group	Individual	Group	Individual	Individual	Individual	Group	Group	Group
Behaviours:										
Agressiveness	⇓ NS	⇓ NS	–	/	–	–	–	/	⇓ ***	⇓ NS
Biting: bars, wire,feeder	–	–	⇓ **	⇓ *	⇑ NS	⇓ NS	⇓ NS	–	–	⇓ *
Inactivity	–	–	⇓ NS	–	⇓ NS	⇓ NS	⇓ NS	–	–	–
Self-grooming	–	–	⇓ NS	–	⇑ NS	⇑ NS	⇑ NS	–	–	⇓ **
Alo-grooming	–	–	–	–	–	–	–	–	–	⇑ **
Sniffing	–	–	⇓ NS	–	⇑ NS	⇑ NS	⇓ NS	–	–	–
Feeding	–	–	⇓ NS	⇑ *	⇑ NS	⇓ NS	⇓ NS	–	–	–
Caecotrophy	–	–	⇓ NS	⇑ *	⇓ NS	⇓ NS	⇑ NS	–	–	–
Drinking	–	–	⇑ NS	–	⇑ NS	⇑ NS	⇑ NS	–	–	–
Contact with neighbour rabbit	–	–	–	–	⇓ NS	⇓ NS	⇑ NS	–	–	–
Sniffing another rabbit	–	–	–	–	–	–	–	–	–	⇑ **
Hopping	–	–	⇑ NS	–	⇓ NS	⇓ NS	⇓ NS	–	–	⇑ **
Stretching	–	–	–	–	⇓ NS	=	⇑ NS	–	–	–
Rearing up	–	–	–	–	⇓ NS	⇓ NS	⇓ NS	–	–	–
Alert	–	–	–	–	–	–	–	–	–	⇓ NS

⇓ - decrease of observed behaviour in enriched environment in comparison with barren environment
⇑ - increment of observed behaviour in enriched environment in comparison with barren environment
= - the same value in barren and enriched environment
/ - behaviour did not occur
– - behaviour was not recorded

NS - not significant
* - $P \leq 0.05$
** - $P \leq 0.01$
*** - $P \leq 0.001$

Table 2. A summary of studies within COST Action 848 of the effects of environmental enrichment on rabbits' performance.

Experiment	Maertens and Van Oeckel (2001)		Jordan and Stuhec (2002)	Luzi *et al.* (2003a)	Jordan *et al.* (2004)			Maertens *et al.* (2004)	Princz *et al.* (2005)	Verga *et al.* (2005)
Enrichment	Wooden stick	Straw in a hopper	Wooden stick (Norway spruce)	Wooden stick (Robinia)	Wooden stick (Oak)	Wooden stick (Lime)	Wooden stick (Norway spruce)	Plastic platform + hiding box + wooden stick	Wooden stick	Wooden stick (Robinia)
Housing	Group	Group	Individual	Group	Individual	Individual	Individual	Group	Group	Group
Performance traits:										
Weight gain (g/d)	⇑ NS	=	⇑ NS	⇑ NS	⇓ NS	⇓ NS	⇓ NS	⇑ NS	⇑ NS	⇓ NS
Feed intake (g/d)	⇑ NS	⇓ NS	−	−	⇓ NS	⇓ NS	⇓ NS	=	⇓ NS	−
Feed conversion ratio (g/g)	=	⇓ NS	−	−	=	⇓ NS	⇓ NS	−	⇓ NS	−
Mortality (%)	=	⇓ NS	−	/	−	−	−	⇑ NS	⇓ NS	−

⇓ - decrease of observed performance trait in enriched environment in comparison with barren environment
⇑ - increment of observed performance trait in enriched environment in comparison with barren environment
= - the same value in barren and enriched environment
/ - performance trait did not occur
− - performance trait was not recorded
NS - not significant

and housing" effectiveness and suitableness of particular enrichment for individually and group housed growing rabbits were evaluated by measuring their effect on rabbits' behaviour, production and also carcass and meat characteristics.

3. Effect of environmental enrichment on rabbits' behaviour

The most often used environmental enrichment for group and individually housed growing rabbits was a wooden stick for gnawing made of different types of wood (Table 1). This kind of enrichment proved to be quite appropriate for growing rabbits since it gave them the opportunity to gnaw. This behaviour seems to be, according to observations of rabbits in semi-natural enclosures, a very important activity in their ethogram (Stauffacher, 1992).

Besides giving the animals material to gnaw, the addition of wooden sticks also decreased the frequency of undesired and abnormal behaviour such as aggressiveness and bar or wire gnawing, which is also one of the goals of environmental enrichment. Maertens and van Oeckel (2001), similarly to Huls *et al.* (1991) and Brooks *et al.* (1993), found that rabbits showed an interest in wooden sticks over a longer period of time, although the intake was low (0.07 – 0.32 g/day). Similar results were obtained by Jordan and Stuhec (2002), where the average daily intake per rabbit was 0.14 g.

Although the suitableness of particular environmental enrichment should be evaluated by also measuring what is important to the rabbits, that is, what they prefer (Olsson *et al.* 2003), only Szendrő (2005 – unpublished data) and Jordan *et al.* (2004) tested which tree-type wooden sticks rabbits prefer. Szendrő (2005 – unpublished data) found out that among wooden sticks made from nine different types of tree, rabbits refused sticks made of *Sambucus nigra* (European elder), *Picea abies* (Norway spruce), *Betula pendula* (Silver birch) and *Morus alba* (White mulberry), and preferred sticks made of *Robinia pseudeacacia* (Robinia), *Salix alba* (White willow), *Tilia cordata* (Lime), *Populus nigra* (Black poplar), and *Aesculus hippocastanum* (Horse-chestnut). Among these five preferred types of wood *Tilia cordata* was, according to the volume consumed, the favourite.

On the other hand, Jordan *et al.* (2004), who used gnawing sticks made of *Qercus robur* (oak), lime and Norway spruce as an environmental enrichment found that rabbits spent a greater percentage of time gnawing Norway spruce sticks than sticks made of lime and oak.

4. Effect of environmental enrichment on rabbit production, carcass and meat traits

Besides the absence of abnormal behaviour, one of the sign of the effectivenss of environmental enrichment and improvement in welfare is also an improvement in production (Baumans, 2005). In some studies environmental enrichment did not significantly influence rabbits' performance (Table 2.), however in most of the studies, it was observed that rabbits housed in an enriched environment had a trend to higher daily weight gain and a lower feed conversion ratio and mortality than rabbits housed in a non-enriched environment. The addition of environmental enrichment also beneficially influenced some carcass characteristics of rabbits (Table 3), although most of the differences were not significant. A significant increase of the slaughter weight of rabbits from enriched cages was observed only by Luzi *et al.* (2003a), while a significant increase in dressing percentage was found only by Princz *et al.* (2005). Most of the studies also reported that rabbits from an enriched environment had a greater percentage of fat and liver, but a significant difference in percentage of fat was found only by Princz *et al.* (2005) and in percentage of liver by Maertens and van Oeckel (2001). Luzi *et al.* (2003b) and Kermauner *et al.* (2004) found that the addition of a wooden stick significantly influenced some meat characteristics (Table 4): meat of rabbits reared in an enriched environment was redder (Luzi *et al.*, 2003b, Kermauner *et al.*, 2004), had a paler colour (Kermauner *et al.*, 2004) and higher moisture (Luzi *et al.*, 2003b) than meat from rabbits reared in a barren environment. All these results suggest that the addition of a wooden stick as environmental enrichment has a slight but mostly positive effect on productive traits and carcass and meat characteristics of rabbits.

Conclusions

Summarizing the information from the literature, it can be concluded that the results of the presented studies point out some common beneficial trends of environmental enrichment influences on rabbits' behaviour, production, carcass and meat quality. At this point it should also be stated that environmental enrichment is not important only from the animals' point of view but also from the farmers'. Besides the fact that improved rabbit welfare could give increased financial returns by boosting growth rate or feed conversion efficiency, the introduction of environmental enrichment to rabbit farming could also improve the public image of intensive breeding systems.

Table 3 A summary of studies within COST Action 848 of the effects of environmental enrichment on rabbits' carcass characteristics.

Experiment	Maertens and van Oeckel (2001)		Jordan and Stuhec (2002)	Luzi *et al.* (2003a)	Kermauner *et al.* (2004)			Princz *et al.* (2005)	Verga *et al.* (2005)
Enrichment	Wooden stick	Straw in a hopper	Wooden stick (Norway spruce)	Wooden stick (Robinia)	Wooden stick (Oak)	Wooden stick (Lime)	Wooden stick (Norway spruce)	Wooden stick	Wooden stick (Robinia)
Housing	Group	Group	Individual	Group	Individual	Individual	Individual	Group	Group
Carcass trait:									
Slaughter weight (g)	⇑ NS	⇑ NS	⇑ NS	⇑ **	⇓ NS	⇓ NS	⇓ NS	⇑ NS	⇓ NS
Carcass weight (g)	⇑ NS	⇓ NS	−	⇑ **	⇑ NS	⇓ NS	⇓ NS	−	−
Dressing percentage (%)	⇑ NS	⇓ NS	−	=	⇑ NS	⇓ NS	⇓ NS	⇑ *	−
Drip loss (%)	−	−	−	−	⇓ NS	⇓ NS	⇓ NS	−	−
Liver (%)	⇑ *	⇑ NS	−	−	⇑ NS	⇑ NS	⇓ NS	−	−
Kidneys (%)	−	−	−	−	⇑ NS	⇑ NS	⇑ NS	−	−
Weight of digestive organs (g)	−	−	⇑ NS	−	−	−	−	−	−
Kidneys fat (%)	−	−	−	−	⇑ NS	⇓ NS	⇓ NS	−	−
Perirenal fat (%)	−	−	−	−	−	−	−	⇑ NS	−
Scapular fat (%)	−	−	−	−	−	−	−	⇑ *	−
Fat (%)	⇑ NS	=	−	−	−	−	−	−	−
Fore part (%)	−	−	−	−	−	−	−	⇑ NS	−
Intermediate part (%)	−	−	−	−	−	−	−	⇓ NS	−
hind part (%)	−	−	−	−	−	−	−	⇓ NS	−

⇓ - decrease of observed carcass trait in enriched environment in comparison with barren environment
⇑ - increment of observed carcass trait in enriched environment in comparison with barren environment
= - the same value in barren and enriched environment
− - carcass trait was not recorded
NS - not significant
* - $P \leq 0.05$
** - $P \leq 0.01$

Table 4. A summary of studies within COST Action 848 of the effects of environmental enrichment on rabbits' meat characteristics.

Experiment	Maertens and van Oeckel (2001)				Luzi *et al.* (2003a)		Kermauner *et al.* (2004)					
Enrichment	Wooden stick		Straw in a hopper		Wooden stick (Robinia)		Wooden stick (Oak)		Wooden stick (Lime)		Wooden stick (Norway spruce)	
Housing	Group		Group		Group		Individual		Individual		Individual	
Meat quality trait:												
pH_{24}	−		−		−		⇓	NS	⇑	N	⇑	NS
Lightness (CIE L*)	⇑	NS	⇓	NS	−		⇑	NS	⇑	*	⇓	NS
Redness (CIE a*)	−		−		⇓	*	⇑	NS	⇓	N	⇓	*
Yellowness (CIE b*)	−		−		−		⇑	NS	⇑	N	⇓	NS
Moisture	−		−		⇑	*	−		−		−	

⇓ - decrease of observed meat quality trait in enriched environment in comparison with barren environment
⇑ - increment of observed meat quality trait in enriched environment in comparison with barren environment
− - meat quality trait was not recorded
NS - not significant
* - $P \leq 0.05$

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2.10. Group size and stocking density

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Introduction

Several experimental results have been published on the effect of group size and stocking density on the performance of growing rabbits. These results will be divided into 5 groups:

- single caged rabbits,
- stocking density in cage,
- group size in cage,
- stocking density in pen,
- comparison of rabbit performance in cages and pens.

1. Single caged rabbits

In Latin European countries rabbits are mainly kept in cages either individually or in groups of variable size (Morisse and Maurice, 1996). Maertens and De Groote (1984) and Xiccato *et al.* (1999) studied the productive performance of growing rabbits housed in individual or in collective cages (four or three animals/cage) at similar densities (15.4, 12 or 16 animals/m^2). Rabbits kept in groups showed lower feed intake (7 %), weight gain (4 - 6 %) and final live weight (3 - 4 %), while feed conversion and mortality were similar. Investigating the carcass traits (Xiccato *et al.*, 1999), the dressing out percentage and meat/bone ratio was also similar in both groups. In group housed rabbits the meat was also paler and cooking losses were higher, as was the diameter and fracture resistance of the tibia.

Individual housing gives an advantage to breeders with its higher productivity, with less contamination among animals resulting in a lower level of infection, diseases, and mortality and avoids the risk for aggressive behavior. But it must also be considered that a lack of social contact among animals may cause stress and this is contrary to animal welfare criteria.

2. Stocking density in cage

The main question is: what is the highest stocking density which is not contrary to welfare and productive performance?

The effect of stocking density was examined in different sized cages (0.21 - 0.66 m^2), with different numbers of animals per cage (from two to ten) and with different stocking densities (9.6 - 28.2 animals/m^2) as shown in Table 3 at the end of this subchapter.

Growth rate declined when the density was higher than 15.4 - 15.6 animals per m^2 (Maertens and De Groote, 1984, Coulmin *et al.*, 1982), or than 19.8 animals/m^2 (Aublet and Duperray, 1992). According to these results, the effect of stocking density depends on cage size and on the final weight (age) of the rabbits. In the experiment of Maertens and De Groote (1984) the cage was smaller and the animals were older (77 days) and heavier (2.4-2.5 kg) than in the trial carried out by Aublet and Duperray (1992) (68 days and 2.2-2.4 kg). If the density (animals or kg/m^2) was lower or the cages were larger (Table 1), then density did not influence the growth rate.

The total weights of rabbits per m^2 were calculated in relation to the density and the age of rabbits. If the total weight of rabbits per m^2 reaches about 40 kg (Maertens and De Groote, 1984) or 46 kg (Aublet and Duperray, 1992) a negative influence on weight gain is expected. This is because the effect of density is weaker at a younger age and is stronger in older animals.

Table 1. Experimental designs for examining the effect of stocking density.

Authors	Size of cage/pen (m^2)	Number of animals/cage or pen								
		2	3	4	5	6	7	8	9	10
		Stocking density, animals/m^2								
Eiben *et al.*, 2001	0.16	12.1	18.2							
Verga *et al.*, 2004	0.21	9.6	14.3	19.2						
Maertens & De Groote, 1984	0.26		11.6	15.4	19.3	23.2				
Xiccato *et al.*, 1999	0.25 - 0.29		12-16							
Coulmin *et al.*, 1982	0.32			12.5	15.6	18.7				
Aubret & Duperray, 1992	0.35					16.9	19.8	22.6	25.4	28.2
Maertens & De Groote, 1985	0.46						15.4	17.5	19.7	
Eiben *et al.*, 2001	0.47						13.9		18.2	
Trocino *et al.*, 2002	0.50 - 0.66							12-16		

Growth rate is closely connected to feed intake and body weight of the rabbits. In most cases the stocking density did not influence feed conversion (the higher the feed intake, the higher the weight gain). But Trocino *et al.* (2004) observed higher feed efficiency for groups of higher density. Mortality was independent of stocking density (Maertens and De Groote, 1984, 1985, Aubret and Duperray, 1992). Rearing rabbits at a high density had scarce effects on dressing out percentage (58.7 *vs* 57.8 %) and on meat red index (Xiccato *et al.*, 1999).

Aubret and Duperray (1992) and Maertens and De Groote (1984) observed fur plucking and ear biting in several high density cages. These problems could be related to the age (sexual maturity) of the rabbits.

Matics *et al.* (2004a) have shown that rabbits weaned at an early age (21 d) like to huddle together. In a free choice experiment the weaned rabbits choose one of the smallest cages where the stocking density could reach 50 - 70 rabbits/m^2. Basing on this observation and on the optimal density in weight of rabbits/m^2 and Matics *et al.* (2004b) studied the two-phase rearing system, housing double the number of animals per cage between 3 and 6 weeks of age and halving the groups afterwards. According to the results no difference was found in the productive traits compared to the control group. Similar results were published by Samoggia *et al.* (1988).

In accordance with the scientific results, in cages where double density is applied during the first phase of the fattening period there is no negative effect on productive performance and behavioural patterns compared to rearing rabbits at up to 16 - 19 animals/m^2 (40 - 45 kg weight/m^2) during the whole fattening period, according to the final weight (age).

The free choice observation showed that early weaned rabbits prefer staying together in a small cage with high density (Matics *et al.*, 2004). It was also demonstrated in free choice tests with wire net *vs.* deep litter in one pen that at an average density of 16 rabbits/m^2, the number of animals per m^2 was 27 - 28 on wire netting, while only 4 - 5 on deep litter (Orova *et al.*, 2004). After weaning, rabbits prefer staying on a plastic netting floor with a stocking density of 38 animals/m^2 (Matics *et al.*, 2003). However, at the end of the fattening period similar densities were found on plastic slat, plastic netting and wire netting floor. These results show rabbits tended to group together at a higher density instead of opting for better living conditions.

3. Group size in cage

Nowadays the public pay more attention to social behaviour. In intensive rabbit housing systems the breeders' demands and the animals' welfare are taken into consideration. This is why the performance in small (2 - 3 animals/cage) and larger cages (6 - 8 animals/cage) were compared. In some experiments the stocking density was similar but the cage size (number of animals/cage) was different (Mirabito *et al.*, 1999a, b, Luzi *et al.*, 2000). In some other trials both the group size and the stocking density were different (Eiben *et al.*, 2001, Princz *et al.*, 2005a). Princz *et al.* (2005b) examined the cage and the pen systems as well (Table 2).

The group size (2 or 6 rabbits/cage) did not affect the weight gain, body weight and the feed intake in the experiments of Mirabito *et al.* (1999b) and Luzi *et al.* (2000). Slightly better results were achieved by Mirabito *et al.* (1999a) in the cages with six rabbits but these fluctuated depending on the

repetition. In other experiments neither the group size nor the stocking density affected the weight gain, body weight, feed intake and mortality (Eiben *et al.*, 2001, Princz *et al.*, 2005a). It seems that if the stocking density is lower than recommended (Maertens and De Groote, 1984, Aubret and Duperray, 1992) the group size has no effect on productive traits. This statement was confirmed by Rommers and Meijerhof (1998) who compared different group sizes (6 - 12 - 18 - 30 - 42 - 54/cage) with the same stocking density (17/m^2).

Dressing out percentage was not influenced by group size (Mirabito *et al.*, 1999a, Luzi *et al.*, 2000, Princz *et al.*, 2005a). The amount of peri-renal fat decreased with the increasing number of rabbits per cage (Princz *et al.*, 2005a).

The percentage of ear lesions was correlated with group size (6, 7.8, 8.1 and 17.4%, Princz *et al.*, 2005a). In two similar experiments Bigler and Oester (1996) observed a higher number and more serious injuries on the animals as a result of group size but, in contrast, Rommers and Meijerhof (1998) reported no influence of group size on the frequency of body injuries. It seems that the increase of group size is limited by the aggressive behaviour in connection with the final age (sexual maturity).

Table 2. Experimental designs for examining the effect of group size.

Authors	Size of cage/pen (m^2)	Number of animals/cage or pen									
		2	3	6	7	8	9	10	13	20	26
		Stocking density, animals/m^2)									
Luzi *et al.*, 2000	0.12	16.7[1]									
	0.36			16.7[2]							
Mirabito *et al.*, 1999a,b	0.21	18.4 - 19.5									
	0.62 - 0.89			17.4							
Eiben *et al.*, 2001	0.16	12.1	18.2								
	0.48				13.9		18.2				
Princz *et al.*, 2005	0.12	16									
	0.50			12		16					
	0.86							12	16		
	1.72									12	16

[1] indoor, [2] outdoor

4. Stocking density in pens

In most of the experiments the stocking density was lower in pens than in cages. The effect of stocking density on productive performance in pens was studied only in a few experiments.

Lambertini *et al.* (2001) reared rabbits in 1 m^2 pens on straw litter at two densities (8 or 16 animals/m^2) in experiment one or in the same sized pens (1 m^2) using the same densities to experiment one (8 or 16 animals/m^2) on straw or on sawdust (experiment two). In the first experiment no difference was observed in the production of the two groups. However in the second the growing performance of rabbits kept at a lower density was higher but feed intake and feed conversion had similar values. In experiment one, mortality was higher for rabbits reared at the higher density (15.6 *vs* 4.2 %) and its incidence was mainly due to coccidiosis.

Maertens *et al.* (2004) housed rabbits in barren pens at a high stocking density (17.9 rabbits/m^2) or in enriched pens (with plastic platform, hiding box, gnawing material) at a low density (8.9 rabbits/m^2). During the first two weeks, feed intake and weight gain were higher in the enriched pens but in the following weeks the performances were similar.

Princz *et al.* (2005a) examined the effect of density (8, 12 or 16 animals/m^2) in pens (0.82 m^2) with different flooring (wire net or deep litter during the whole period or the wire net was covered by straw two or four weeks after weaning). The stocking density had a slight (non significant) effect on weight gain, live weight and feed intake (higher values in group of higher density) but the feed conversion and mortality were the same in both groups. A similar experiment was carried out by Kustos *et al.* (2003) with the density of 5, 8.7, 12.5 and 16.2 animals/m^2, in pens of 0.8 m^2. In this case the productive traits were affected by the stocking density, the performance in the groups of higher densities mostly decreased during the second part of the experiment. Lambertini *et al.* (2001) and Maertens *et al.* (2004) did not observe any aggressive outbursts among animals or injuries on them.

Carcass traits were not affected by the density, the pH value; color and chemical composition of meat were the same in both groups (Lambertini *et al.*, 2001).

As the results show, the stocking density in pens up to 16 rabbits/m^2 has only a slight effect on the

Table 3. Experimental designs to compare the cage and pen housing systems.

Authors	Cage			Pen				
	Size (m^2)	Animals per cage	Stocking density	Size (m^2)	Animals per cage	Stocking density	Floor type	Top
Combes *et al.*, 2003	0.38	6	15	0.66[1]	10	15	Wire net	Yes
				4.05	60	15	Wire net	No
Van der Horst *et al.*, 1999	0.44	7	16	8.00[2]	64	8	Wire net	?
Jehl *et al.*, 2003	0.35	6	17	2.64	45	17	Wire net	No
	0.59	10	17					
Maertens and Van Oeckel, 2001	0.26	4	15	1.90	30	16	Wire net	No
Maertens and van Herck, 2000	0.26	4	15	1.90	30	16	Wire net	No
Princz *et al.*, 2005	0.12	2	16	0.83	13	16	Wire net	No
Metzger *et al.*, 2003		3	19		80	8	Straw	No
Dal Bosco *et al.*, 2002	0.12	2	16.6	1.23	12	10	Wire net	?
				1.23	12	10	Straw	
Dal Bosco *et al.*, 2000	0.12	2	17	1.23	12	10	Straw	?
Lambertini *et al.*, 2001	0.13	2	16	1.00	8	8	Straw[3]	
				1.00	16	16	Straw[3]	
	0.13	2	16	1.00	8	8	Wood shavings	
				1.00	16	16	Wood shavings	
				1.00	8	8	Straw	
				1.00	16	16	Straw	

[1] Elevated platform
[2] In - and outdoor
[3] Males and females separately

production of rabbits. But it should be noted that the mortality rate could be higher on deep litter at a higher density if the feed is not supplemented with medication.

5. Cage *vs* pen

Modern consumers are interested in meat of high quality produced under conditions of a high level of animal welfare. This fact stimulates researchers to study alternative systems, which can offer more natural conditions allowing the rabbits a wide range of behaviour patterns (Ferrante *et al.*, 1992, Mirabito *et al.*, 1999b, Morisse *et al.*, 1999).

Many investigations have been carried out to compare cage and pen housing systems. The experimental designs of the trials are summarized in Table 3.

Comparing the performance of rabbits housed in cages or pens we have to discuss floor types separately (wire net or deep litter). Compared to cage housed rabbits in pens, the feed intake, weight gain and live weight decreased by 3 - 7, 2 - 8 and 3 - 8% respectively if the floor was wire netting, while the decrease was significantly higher (12 - 18, 17 - 23 and 12 - 16 %, respectively) in the case of deep litter. Several authors observed litter consumption. The lower feed intake of rabbits on a straw bed compared to those on wire netting could be due to the consumption of straw litter.

The reduction of growth rate in the pen housing systems could be explained by the greater physical activity and/or the lower feed intake (Dal Bosco *et al.*, 2002). The higher mortality rate in pens could be due to increased infectious pressure caused by the larger group size (Maertens and van Herck, 2000). In rabbits reared on a straw-bedded floor, the contact with excrement and its consumption with the straw increase the risk of contamination and coccidiosis and leads to health problems, which could be the primary cause of the diminished production (Lambertini *et al.*, 2001).

The percentage of resting was lower in rabbits housed in pens (Dal Bosco *et al.*, 2002). The higher level of activity is generally said to be the expression of good welfare. But we should also take note of the aggressive behaviour. The frequency of ear lesions was higher in pen housed rabbits (Princz *et al.*, 2005b). The frequency of lesions induced by aggressive behaviour can be reduced by inserting a gnawing stick into the cage/pen (Princz *et al.*, 2005b).

In large groups the risk of contamination and aggressiveness is higher. At the temperature of 15 - 20 °C rabbits prefer staying on wire netting instead of deep litter (Orova *et al.*, 2004). The risk for enteric diseases, especially coccidiosis, is high on litter because of the consumption of litter material and the direct oral contact with faeces. The decrease in the production of growing rabbits reared in large groups is 3 - 4 times higher on deep litter compared to wire netting floors. Summarizing the advantages and disadvantages of rearing rabbits in smaller or larger groups on wire netting or deep litter, the following can be concluded: raising growing rabbits in pens on wire netting floors, at a moderate density, in medium large groups (e.g. a whole litter together) and using wooden sticks to reduce body lesions caused by aggressive behaviour seems to be a good alternative housing system.

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2.11. Animal protection in housing and transport

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1. European and national legislation concerning housing of rabbits

Although there are no common European rules on rabbit welfare, apart from laboratory animals (EC Directive 86/609, 1986), in many European Countries local guidelines on rabbit housing systems exist. Codes of Welfare for the housing systems and management of many species, including rabbits, have been laid down since 1987, revised in 1989, for example in the U.K. (Home Office, 1989). Furthermore, in the UK, Guidelines on the housing and care of rabbits have also been issued by the Ministry of Agriculture, Fisheries and Food (MAFF 1971, 1990), the Government Statutory Instrument 2000 n 1870, "The welfare of Farmed Animals (England) Regulations" (2000) and the Laboratory Animal Breeders' Association (LABA, 1991). These Codes include indications on the space requirements for the animals, reared both in cages and on the ground (Table 1) (Verga, 2000), taking into account that the total floor area should be sufficient to allow the rabbits to move around and feed and drink without difficulty. Accommodation should allow sufficient area and height to permit the rabbits to lie on their sides and to sit down. Finally, the nest should be large enough to permit the doe to go into and out to feed the kits without injuring them (Animal Welfare, Rabbit Welfare Code, Government UK, 1987). Whatever system of housing animals is employed, a good management system is important.

The German section of the WRSA (World Rabbit Science Association) has issued (1991) some indications on the minimum space related to the rabbits to intensive housing systems (Verga, 2000) (Table 2). Furthermore, guidelines have also been published in Switzerland (Swiss Order on Animal Protection, 1981, Stauffacher, 1992, Bigler and Oester, 1996).

Table 1. Minimum space allowance for rabbits (According to Home Office, Animal Welfare, Rabbit Welfare Code, 1987).

System	Minimum Floor Space
In Cages	
Doe and litter to 5 weeks of age	0.56 m^2 total area
Doe and litter to 8 weeks of age	0.74 m^2 total area
Rabbits 5 to 12 weeks of age	0.07 m^2 per rabbit
Rabbits 12 weeks and over[1]	0.18 m^2 per rabbit
Adult does and bucks for rearing	0.56 m^2 per rabbit
In Hutches	
Doe and litter to 5 weeks of age	0.75 m^2 total area
Doe and litter to 8 weeks pf age	0.93 m^2 total area
Rabbits 5 to 12 weeks of age	0.09 m^2 per rabbit
Adult does and bucks for breeding	0.75 m^2 per rabbit

[1]other than those used for rearing (multiple occupation cages)

In Italy, two sets of legislation exist concerning the welfare of farm and laboratory animals (D.Lgs. 26-3-2001 n. 146; D.DLT 27/01/1992 n. 116).

In The Netherlands, no directives on rabbit housing have yet been issued, but these are in preparation by the Dutch Meat Board (Ruis, 2004).

In Spain, there exists the "Real Decreto 441/2001 de 27 de Abril" (Real Decreto 348/2000 de 10 de Marzo – EC Directive 98/58) in relation to animal protection in farms using intensive rearing, without specific mention of the rabbit species.

In Greece, as in other European countries, to date there have been no specific directives concerning rabbit housing; however in the future it is expected that the European Community Directives will be in common use.

Table 2. Indications for rabbits' housing by W.R.S.A. (According to German Section, 1991).

	Minimum space (m^2)	Height (cm)
Breeders		
Up to 4 kg live weight	0.20	35
Up to 5.5 kg live weight	0.30	40
More than 5.5 kg live weight	0.40	40
Growing rabbits		
From weaning to 6 weeks	0.04	
Up to 3.3 kg live weight	0.08	35
Pens	0.12	35
Nest	0.10	30

2. General considerations, European and national legislation concerning transport of rabbits

It is important to note that the word "transport" is defined as the entire process from the time the first animal is loaded until the last animal is unloaded. The transport of rabbits used for breeding or for meat production is stressful for the animals, especially during loading or unloading. According to a Spanish questionnaire study of rabbit farms (n = 60), lorry drivers (n = 21) and abattoirs (n = 21), there are a series of critical points during transport. These are moments and/or places where the handler should be especially careful since the welfare of the rabbit could be easily compromised. They include waiting at the farm before loading (sometimes longer than the transport itself), the loading system (improved in larger companies), ventilation and temperature during transport (which depends on cage position and environmental conditions of the vehicle), loading stops at other farms (increasing transport time), unloading (with systems that are faster and improved), waiting before slaughter (time and thermo-neutral conditions in the lairage area) and the time between stunning and bleeding (Buil *et al.*, 2004).

Therefore most of the stressors pointed out by Jolley (1990) could come into play during this period. However, more studies are needed to measure how each of the different critical points contribute to increasing stress, which would help improve commercial practice. The adverse effects of transport and ante-mortem conditions on live weight or carcass output are already known (Luzi *et al.*, 1992, Ouhayoun and Lebas, 1994), though the transport itself cannot be considered as a 'macrostressor' according to Finzi and Verità (1980). Temperature, both in laboratory trials and commercial transport, is a very important factor with regard to welfare, since it affects stress, muscular fatigue and dehydration, both when it gets too hot or too cold (De la Fuente, 2004). According to these authors, rabbit density in the cages and transport time are less important. The position of the cage on the truck also affects the welfare of the rabbits, being worse in the middle or lower positions (Liste *et al.*, 2004). At the European level, both the former scientific committee on animal health and animal welfare (SCAHAW; website EC, 1991, 1993, 1998, 2003, 2005 http://europa.eu.int/comm/food/animal/index_en.htm) and the EFSA (European Food Safety Authority) Panel on animal health and animal welfare (http://efsa.eu.int) have produced reports and opinions on animal welfare during transport.

All the European Countries apply the EU directive 91/628/EEC on animal transport and in some countries National regulations exist.

In England there is the Statutory Instrument 1997 n° 1480 "The Welfare of Animals (Transport) Order 1997", which, in article 4, deals with transport of poultry and domestic birds and domestic rabbits (1997).

Table 3. Stocking densities for fattening rabbits during transport (immature rabbits with a maximum age of 90 days, transported for further fattening or for slaughtering not longer than 12 hours) (According to German law on animal transport from 25 February1997, changed in 2002).

Live weight up to (kg)	Height of the transport box (cm)	Space per rabbit (cm^2)
1	15	250
3	20	500
>3	25	600

The German national regulations established the stocking densities shown in Tables 3 and 4. Fattening rabbits are mainly transported in Germany in (poultry) containers measuring 60 x 80 x 25 cm with 8 rabbits per container (600 cm^2 per rabbit).

The floor of containers is perforated. The duration of the journey is a maximum of four hours (with loading, waiting and unloading at a maximum eight hours). The rabbits are transported with an open roof in good weather or with a covering in case of adverse weather. Breeding rabbits are transported in smaller containers, normally measuring 60 x 40 x 25 cm with two rabbits per container (1000 – 1200 cm^2 per rabbit). The containers have a compact (closed) floor with raised wire netting to prevent contact with urine and faeces. The maximum duration of the journey in closed air-conditioned cars 12 hours (Hoy, 2004).

Table 4. Stocking densities for other rabbits during transport (According to German law on animal transport from 25 February1997, changed in 2002).

Live weight up to (kg)	Height of transport box (cm)	space per rabbit (cm^2)	maximum number of rabbits per box
0,3	15	100	12
0,4	15	150	12
0,5	15	300	12
1	20	500	4
2	20	750	4
3	25	900	2
4	25	1000	2
5	25	1150	2
>5	30	1400	1

Hungary has two national regulations, the 243/1998-XII. 31 (Government directive on animal test) deals with the temperature requirenments of rabbits (12 – 24 °C) and the 52/2203-VIII. 15 (Common directive of the Ministry of Economy and Transport and the Ministry of Agriculture on animal transport) reports that rabbits placed in containers during transport can be transported in vehicle without sides. The volume of rabbit transport is low. Transport is mainly of short distance and duration. The maximum distance from farmer to slaughterhouse is approximately 200 km (Szendrő reported by Luzi *et al.*, 2004).

In Greece, there exist only two large-scale farms with their own slaughterhouses. Here, there is the problem of heat stress under hot climatic conditions. Greece has no national regulations regarding rabbit transportation. (Xyloury reported by Luzi *et al.*, 2004).

In Spain, 125 million rabbits were transported in 2001. In Spain, the "Real Decreto 1041/1997 de 27 de Junio" is in force, which establishes the directives for animal protection during transport. Regarding the transport of rabbits, it is reported that animals need to be provided with feed and drink when the duration of transport is more than 12 hrs, excluding the loading and unloading periods (Levrino, 2004).

In Italy, the number of fattening rabbits per farm is on the increase and slaughterhouses will also become larger in size but fewer in number, so that distances between farm and slaughterhouse will probably become longer in the near future. There are no special regulations concerning rabbit transport in Italy, but it has acknowledged the 95/29/CE directive on animal transport protection (DL 20-10-1998 n. 388). The statistics on rabbit transport are based on the total weight (in tons) of all slaughtered animals (222,000 Mt in 2004: FAO Statistical Database). The importance of seasonal conditions (especially at high temperatures combined with high humidity) is underlined (Finzi reported by Luzi *et al.*, 2004).

Belgium has four slaughterhouses with a capacity around 10,000 rabbits per week, so that good statistics (on the basis of weight and numbers of slaughtered animals) are available. There is a lack of specific reports and results regarding rabbit transport (Maertens reported by Luzi *et al.*, 2004).

The Netherlands have no specific regulations, but there is a good "Integrated Chain Control" including data on rabbit transport. To date there has been no research in the field of rabbit transport in The Netherlands (Ruis reported by Luzi *et al.*, 2004).

3. Conclusions

It can be concluded that

- all European Community Countries apply EC Directive 91/628/EEC; however, certain countries (i.e. German, Spain) have even stricter National Regulations,
- some data on rabbit transport (stocking density, number of animals, etc.) are available, but not in all European Countries and not for breeding animals,
- some reports of results and practical experiences concerning rabbit transport exist but these vary widely from one country to another,
- it has become really necessary to intensify research work on rabbit transport.

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Chapter 3

PATHOLOGY

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Pathology is a major component limiting rabbit production in industrial rabbit farms. It can be approached from the angle of specific diseases: a germ = a disease. This will mostly be done in the different sub-chapters of this section on pathology because it is of course easiest to handle. However, it should not be overlooked that the development of a disease is more complex and that favouring factors can be involved. Some of the most important are: environment, food (Peeters *et al.*, 1993, 1995; Gidenne et Licois, 2005), stress, hygienic standards in breeding…, or other factors specific to the animal itself, such as: genetics predisposition, immunological status or sanitary status (Bennegadi *et al.*, 2001).

Two main syndromes are classically recognized in rabbits: respiratory syndrome which prevails in adults, and digestive syndrome, more frequent in growing rabbits. In addition, pathology related to reproduction in industrial rabbit farms (pathology in the nest or in the females themselves), which is not generally associated with infectious phenomena (Lebas *et al.*, 1996), must be considered but will not be developed in this section.

According to Boucher (unpublished data), 26% of the rabbits analyzed for pathological conditions in France in 2005 had respiratory problems. The clinical signs and lesions vary from nasal congestion and rhinitis to pneumonia. Pasteurellosis is the major recurrent bacterial disease affecting the respiratory system, in which environmental factors can play an important role. A complete chapter is devoted to this pathology, which covers various forms. The well known myxomatosis is not a true respiratory pathology but secondary infections can cause nasal congestion, and the amyxomatous form of the disease generally affects the respiratory tract. Another viral pathology, Viral Haemorrhagic Disease, is characterized by acute pulmonary and hepatic infections. Separate chapters will be dedicated to each of these two contagious and often fatal viral diseases.

Staphylococcosis is also a recurrent disease which can have dramatic effects on commercial breeding in severe cases. New insights on the virulence factors of *S. aureus* are given in this book.

Digestive disorders are responsible for mortality and significant morbidity characterized by reduced growth and poor feed conversion, which often causes greater commercial losses than mortality. The aetiology of intestinal affections is still difficult to establish because the causes are often multiple and symptoms, clinical signs and intestinal lesions are often comparable, but one of the clinical signs, diarrhoea, is largely dominant and may be encountered in more than 95% of cases. In industrial fattening rabbit farms, until the appearance of Epizootic Rabbit Enteropathy (ERE), the main pathogenic agents associated with these digestive pathologies were primarily of parasitic origin (*Eimeria* spp,) and/or bacterial origin (chiefly, enteropathogenic *Escherichia coli*; sometimes *Clostridium spiroforme* and *Klebsiella* and exceptionally, *Clostridium piliforme*). Some viruses

can be observed, but their role as primary pathogens remains questionable. The remaining cases of diarrhoea which cannot be ascribed to a precise aetiology are generally gathered together under the term of "non specific enteritis" (Bennegadi *et al.*, 2000).

Considering the advances of the last few years, only ERE, Colibacillosis and enterotropic virus will be developed here. While coccidia are considered as true pathogens and coccidiosis represent the main parasitological infection in rabbit units, there is no recent data on this topic. However further information can be found in the literature (Coudert *et al.*, 1995; Eckert *et al.*, 1995, Licois, 2004).

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3.1. Recent advances in rabbit staphylococcosis research

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1. Introduction

In rabbits, *Staphylococcus aureus* bacteria can infect small skin lesions and invade subcutaneous tissue. On the individual level, all rabbit *S. aureus* infections lead to similar lesions of pododermatitis ("sore-hocks"), subcutaneous abscesses and mastitis (Okerman *et al.*, 1984; Rossi *et al.*, 1995; Devriese *et al.*, 1996). Suckling kits may suffer from a pustular dermatitis and often die as a result of agalactia in mastitic does. Sporadically, abscesses in lungs, liver and uterus are observed as well, leading to poor production results, infertility and death. Abscesses in broiler rabbits lead to increased slaughterhouse condemnations.

At rabbit flock level, two types of *S. aureus* infections can be distinguished. The first type is caused by so-called low virulence (LV) strains and is of minor commercial importance, as it is limited to individual animals. The second type of infection, caused by high virulence (HV) strains, leads to chronic problems and a decline in production as the infection spreads throughout the rabbitry.

This literature review emphasizes the recent advances in the research concerning epidemiology, pathogenesis and control of staphylococcosis in rabbits.

2. Etiology and epidemiology

The etiological agent of staphylococcosis in rabbits is *Staphylococcus aureus*. This species can be divided into several biotypes on the basis of biochemical properties of the isolates. Some biotypes (so-called ecovars) are considered host-specific, while other may colonise different hosts. Human, bovine, ovine and poultry ecovars have been described (Devriese, 1984). Strains of these ecovars may occasionally be found in other host species.

LV *S. aureus* strains causing infections in rabbits may belong to the human or poultry biotype (Devriese *et al.*, 1981; Devriese, 1984). HV outbreaks of the disease in rabbitries are however caused by strains belonging to another biotype, the "mixed CV-C" type, which is characterized by the production of beta haemolysin, a type of purple growth on crystal violet agar and the lack of staphylokinase (Devriese, 1984; Devriese *et al.*, 1996). However**,** biotyping is not sufficient to distinguish between HV and LV *S. aureus* strains in rabbitries, since the "mixed CV-C" biotype comprises both HV and LV strains (Devriese *et al.*, 1981). To further distinguish between HV and LV strains, other typing methods are necessary, and several have been used for this purpose.

Phage typing provides further subdivision of *S. aureus* strains through identification of bacteriophages to which the bacterium is susceptible. Phage typing is accomplished using bacteriophages of the international typing set for human *S. aureus* strains (Parker, 1962). *S. aureus* isolates from rabbits are usually susceptible to several of these phages. LV strains may belong to a large variety of phage types (Devriese *et al.*, 1981; Hermans *et al.*, 1999). Strains isolated from HV epidemic outbreaks in rabbitries are usually sensitive to phages 3A, 3C, 55 and 71 of phagegroup II. However, a number of strains isolated in the past three years displayed a slightly different biotype, still being sensitive to phages of phagegroup II, but to a number of other phages as well (Vancraeynest *et al.*, 2004a). On one occasion, an HV strain has been described as belonging to the biotype-

phagetype combination "mixed CV-C" - 29/79/42E/92/D11/HK2 (Devriese *et al.*, 1996).

Besides these phenotypic properties, *S. aureus* strains from rabbits have also been examined genotypically. Hermans *et al.* (2000) described that strains of the typical HV pathotype also belong to a specific Randomly Amplified Polymorphic DNA (RAPD) type, which is designated as RAPD type a. Their studies suggested a single clonal origin of the typical HV strains (Hermans *et al.*, 2000; Hermans *et al.*, 2001). This clonal origin was confirmed by a pulsed-field gel electrophoresis (PFGE) study performed by Vancraeynest *et al.* (2006a). By using PFGE, which is now considered to be the method of choice for typing *S. aureus* strains, they demonstrated that all HV rabbit *S. aureus* strains, sensitive to phages of phagegroup II, are clonal. This pathotype has been responsible for severe problems in many European countries, including Belgium, the United Kingdom, Germany, Ireland, Italy, Greece, France and Spain (Okerman *et al.*, 1984; Carolan, 1986; Holliman and Girvan, 1986; Rossi *et al.*, 1995; Hermans *et al.*, 2000) since at least 1983 and its importance has not appreciably declined since its first description. Severe problems of disseminating staphylococcosis have also been described in the U.S.A. (Hagen, 1963) but the *S. aureus* strains responsible have not been further characterised.

The prevalence of LV *Staphylococcus aureus* strains in rabbits is difficult to estimate. Since these infections are only important for individual rabbits and not for entire rabbit flocks, they are not described in international literature. Nevertheless, such infections may be found in pets and in commercial rabbits (Stein and Walshaw, 1996; Flecknell, 2000). Disseminating problems of staphylococcosis have been considered important in Western Europe since the 1980s, and outbreaks have frequently been reported in several European countries (Devriese *et al.*, 1981; Okerman *et al.*, 1984; Carolan, 1986; Holliman and Girvan, 1986; Devriese *et al.*, 1987; Rossi *et al.*, 1995; Devriese *et al.*, 1996). This is probably to the consequence of a higher occupation density due to the appearance of large-scale commercial rabbitries. Because of the increased demand, the traditional production of rabbits has evolved to an industrial or semi-industrial level. High densities of animals increase the seriousness of infectious diseases, since the pathogenic agents have a higher possibility of spreading throughout the flock.

Disseminating staphylococcosis can be found in hygienic as well as in less hygienic rabbitries (Hermans *et al.*, 1999). This might indicate that bacterium-host interactions and not management factors determine the epidemic spread of disease in the rabbitry. It has been shown that HV strains have a higher capacity for colonising host epithelia (Hermans *et al.*, 1999). A greater degree of colonisation may imply a higher infection pressure, which induces the disease to spread throughout the flock.

Transmission of HV and LV *S. aureus* strains from man to rabbit or between rabbits may be direct or indirect, through cages, hairs or food (Devriese *et al.*, 1987; Rossi *et al.*, 1995; Matthes, 1995). Direct transmission of *S. aureus* bacteria may occur between does and suckling young (Devriese *et al.*, 1981; Matthes, 1995), between litter mates and between stable mates (Devriese *et al.*, 1981). Devriese *et al.* (1981; 1987) noticed that rabbitries infected with identical *S. aureus* strains often have either direct or indirect contact, and that intake of new breeding rabbits in the flock is probably the most important source of infection. Sperm (even after artificial insemination) also forms a potential risk of infection by HV *S. aureus* strains in rabbits (Rossi *et al.*, 1995).

3. Pathogenesis

S. aureus bacteria are able to infect small skin lesions. Traumatic lesions may be due to poor quality cage wire floors or to fighting between animals. Pododermatitis due to HV *S. aureus* strains is also found in animals with low body-weight and belonging to breeds known to have a certain resistance to foot-lesions, which may suggest that these virulent strains have a higher ability to cause pododermatitis (Okerman *et al.*, 1984).

Oher possible infection routes may be the umbilical stump in newborn rabbits (Hagen, 1963), the vagina, the preputium or the urethra (Rossi *et al.*, 1995). The mammary gland often becomes infected through suckling young. Several authors (Adlam *et al.*, 1976; Rossi *et al.*, 1995) succeeded in transmitting the bacterium to an uninfected doe by giving her healthy young of an infected doe to foster.

It has been shown that HV *S. aureus* strains from rabbits have a better capacity to colonize the host than LV *S. aureus* strains (Hermans *et al.*, 2000). This may be caused by a difference in adhesive and/or biofilm forming capabilities between HV and LV strains. Therefore, Vancraeynest *et al.* (2004b) performed a phenotypic and genotypic characterization of HV and LV rabbit *S. aureus* isolates for biofilm/slime formation and a genotypic screening for a set of MSCRAMM (microbial surface components recognizing adhesive matrix molecules) genes. This led to the remarkable result that only the typical HV strains were positive for *bbp*, encoding bone sialoprotein binding protein. Further research is necessary to elucidate the significance of *bbp* in the pathogenesis of HV strains.

The pathogenicity of a particular *S. aureus* strain is the result of several factors, including the capacity to produce extracellular protein toxins. To check whether the chronic problems associated with HV *S. aureus* strains are due to a certain toxin produced by

these strains, Vancraeynest *et al.* (2006b) performed a comparison of HV and LV *S. aureus* strains regarding the prevalence of genes encoding exfoliative toxins, leucotoxins and pyrogenic toxic superantigens (PTSAgs). They found that only typical HV strains contain the *egc*-cluster, which is comprised of the genes encoding enterotoxins G, I, M, N and O. As *S. aureus* enterotoxins induce an immunomodulation or even immunosuppression in the host (Schlievert, 1993; Ferens and Bohach, 2000; Fueyo *et al.*, 2005), it is possible that the chronic infections caused by HV rabbit *S. aureus* strains are the consequence of the presence of the *egc* cluster. However, as with *bbp*, further research is necessary to evaluate the role of these genes in the pathogenesis.

Figure 1. *Mastitis in a rabbit, caused by Staphylococcus aureus (Courtesy of Joan Rosell).*

4. Clinical signs

Generally, *S. aureus* infection gives rise to a suppurative lesion or abscess formation at the infection site. Therefore, the lesions caused by *S. aureus* are mainly mastitis (fig. 1) pododermatitis (fig. 2) and subcutaneous abscesses (Okerman *et al.*, 1984). When septicaemia occurs from the primary lesion, abscesses in internal organs, such as liver, lungs and uterus may be formed.

Clinical signs differ depending on the age of the affected animals. Several authors (Okerman *et al.*, 1984; Carolan, 1986; Holliman and Girvan, 1986; Rossi *et al.*, 1995; Matthes, 1995; Devriese *et al.*, 1996). Newborn hairless rabbits may suffer from exsudative dermatitis with superficial pustules. Often, the whole litter is affected and high mortality is seen, especially during the second week of life. In older young, subcutaneous abscesses, conjunctivitis and purulent rhinitis are noticed. Subcutaneous abscesses and pododermatitis frequently occur in broiler rabbits and in does. In rabbits of all age categories, internal abscesses, e.g. in lungs and liver, may be demonstrated. Arthritis, parodontitis, sinusitis and otitis media have also been described. Mastitis in does may vary between an acute gangrenous form ("blue breast") and a chronic form with abscess formation. Agalactia and mortality of the doe may cause a serious increase of the death rate in suckling young. Metritis in does has also been described.

LV strains affect only a limited number of rabbits in the flock. HV strains, on the contrary, cause an epidemic spread of disease in the rabbitry. They give rise to persistent problems of staphylococcosis in commercial rabbitries. Because of agalactia and infertility, increased mortality and decreased growth rate, rabbitries suffering from severe and chronic staphylococcosis are facing substantial commercial losses. Most studies do not mention exact figures on morbidity and mortality. When a rabbitry becomes infected with an HV virulence *S. aureus* strain, the morbidity described varies between 65 percent (Holliman and Girvan, 1986) and 80 percent (Rossi *et al.*, 1995). Mortality in breeding does may reach 35 percent (Holliman and Girvan, 1986). In a study performed by Okerman *et al.* (1984), at least 15 percent of the breeding does had to be replaced before they reached the age of one year, as a consequence of staphylococcosis. Due to staphylococcosis in does, the loss of complete litters after death of their mother is very common (Holliman and Girvan, 1986).

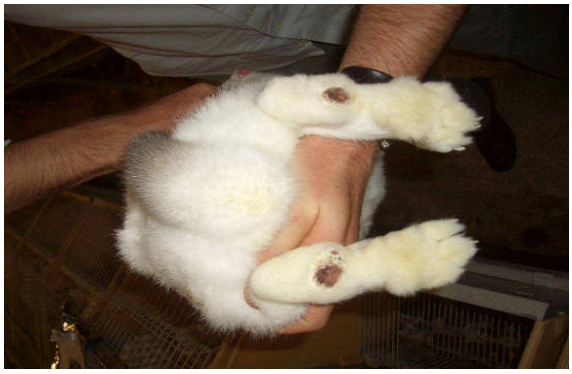

Figure 2. *Pododermatitis in a rabbit, caused by Staphylococcus aureus* (Courtesy of Joan Rosell).

5. Diagnosis

Diagnosis of staphylococcosis is accomplished by isolation of the bacterium from the lesions. After growth on media containing bovine or ovine blood, the *S. aureus* colonies show a typical white to yellow colour. All *S. aureus* strains have haemolytic

properties. Alpha, beta, gamma and delta haemolysin may be produced. Alpha and delta haemolysin provide a clear zone surrounding the bacterial colony. The additional production of beta haemolysin is responsible for an outer zone of incomplete haemolysis or rather a discoloration with sharply demarcated edges. The DNAse test is performed to confirm species identification (Devriese and Hajek, 1980). *S. aureus* is the only DNAse-positive species of staphylococci found in rabbits (Devriese *et al.*, 1981). The distinction between HV and LV strains, which completes the diagnosis, is described in the section on etiology.

6. Control

In rabbits infected with LV *S. aureus* strains, treatment is accomplished by draining and cleaning the subcutaneous abscesses and by antibiotic therapy. This treatment is only performed in pet rabbits, and not in commercial rabbitries (Laval, 1989; Stein and Walshaw, 1996). Although LV strains do not spread throughout rabbitries, they can be responsible for a high rate of pododermatitis among does in wire mesh cages. This problem can be largely resolved by the introduction of atraumatic footrests.

Although HV rabbit *S. aureus* strains do not display a markedly higher antimicrobial resistance level than LV strains (Vancraeynest *et al.*, 2004c), effective antibiotic treatment of rabbit flocks infected with HV *S. aureus* strains is not possible. Thorough cleaning and disinfection of cages and materials, together with antibiotic treatments such as 800 mg tetracycline HCl per kg feed for 7 days, may lead to a decrease of disease and mortality in the flock. However, after stopping the therapy, the problems will arise again (Carolan, 1986; Holliman and Girvan, 1986; Devriese *et al.*, 1987; Rossi *et al.*, 1995). This treatment is not able to eliminate the bacterium from the flock. Culling of diseased or suspected animals, is even less successful (Devriese *et al.*, 1987; Devriese *et al.*, 1996). The only solution after the introduction of HV *S. aureus* strains in a rabbitry is to slaughter the entire flock, to clean and disinfect the building thoroughly, and to start all over again with new rabbits, derived from an unaffected rabbitry.

Prevention of HV staphylococcosis in rabbits is therefore of the uttermost importance, but is a difficult task. The control of staphylococcosis in rabbits might be possible by examination of new animals before their introduction into the flock, for the presence of HV strains. Unfortunately, most rabbits are carriers of *S. aureus* strains. These are predominantly LV strains, however (Hermans *et al.*, 1999). Rabbit carrier sites of *S. aureus* may be the nose, the ear, the skin between the toes and the skin of the foreleg, the axillar and inguinal skin regions, the skin around the nipples, the perineum, the vagina and the preputium. Rabbits may be discretely to highly positive for *S. aureus* on one to nine of these body sites. Therefore, a large number of samples should be taken to avoid false negative results. The perineum seemed to be an important body site for sampling, since it was often highly colonized, although the number of positive samplings was not statistically higher than in other body sites. Because of the high colonization rate of other bacteria on the perineum, it seems useful to inoculate a selective modified Baird-Parker medium (Devriese, 1981), in addition to the non-selective Columbia agar (Gibco, Paisley, Scotland) supplemented with 5 % ovine or bovine blood (Hermans *et al.*, 1999). Bacteriological examination of these carrier sites for the presence of *S. aureus* bacteria before introduction of new rabbits in the flock is not sufficient to prevent intake of the disease. It is necessary to additionally type the isolated strains to distinguish between HV and LV strains and for the prevention of intake of the former strains. Biotyping and phage typing are possible (Parker, 1962; Devriese, 1984), but are time-consuming and more or less difficult to standardise. PFGE is a valuable but expensive technique. Alternatively, RAPD typing can be used, which is a fast and easy-to-perform technique (Hermans *et al.*, 2000b). However, since individual bacterial colonies are necessary for RAPD typing, samples of several body sites of the rabbits should be taken for bacteriological examination, and isolated colonies need to be typed separately, which is a disadvantage for the practical use of the RAPD technique. Moreover, RAPD has a rather low reproducibility in and between laboratories. Thus, efficient tests to achieve control of rabbit staphylococcosis are not available at the present time. A rapid and accurate method, which could also be used on mixed cultures of skin swab samples, would be a PCR-test based on a nucleotide sequence specific for HV rabbit *S. aureus* strains. Therefore, studies on the genomic differences between HV and LV strains are being performed.

Limited introduction of new animals and limited contact between rabbitries may decrease the risk of infection (Devriese *et al.*, 1987; Devriese *et al.*, 1996). Currently, this is the only feasible method of coping with rabbit staphylococcosis.

Vaccination would be another possible method to control rabbit staphylococcosis. Generally, however, immunisation against staphylococcal infections has been unsuccessful (Lee, 1996). Recently, it has been reported that so-called M and N proteins, with a molecular mass of approximately 29 and 27 kDa respectively, are secreted by typical HV strains and absent in LV strains (Hermans *et al.*, 2001b). These proteins can thus be considered as virulence associated markers and may be useful for vaccine development if they are real virulence factors. The same applies to bbp and the enterotoxins encoded by

the genes of the *egc*-cluster. Further study is required to achieve this goal.

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3.2. Myxomatosis

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Introduction-History

Myxomatosis is a major viral disease of wild and domestic European rabbits (*Oryctolagus cuniculus*). The aetiological agent was first isolated from a colony of laboratory rabbits in Uruguay in 1898 and identified as a poxvirus in 1927. The natural hosts are two species of leporid: *Sylvilagus brasiliensis* in South America (South American strains) and *Sylvilagus bachmani* (Californian strains) in California (Fenner, 1994). In its natural hosts, the viral strains produce only a benign fibroma, a generalized disease occurring only in juvenile animals. In European rabbits (*Oryctolagus cuniculus*), the South American and Californian strains of myxoma virus are highly lethal but produce different kinds of disease with generally less prominent clinical symptoms than the Californian strains (Fenner, 1994).

Myxomatosis was first recognised in North America in 1930, where outbreaks of the disease occurred in rabbitries in southern California. Myxomatosis remains enzootic in the western United States, some cases sporadically occurring in domestic rabbits (Patton and Holmes, 1977).

Since 1950, myxomatosis has been used for the biological control of the rabbit pest in Australia. In the initial epidemic in Australia, myxomatosis was estimated to have killed as many as 99.8% of infected wild rabbits and many populations were reduced by more than 90% (Kerr and Best, 1998). Myxomatosis was deliberately introduced into France in 1952 and soon became enzootic throughout Europe (Arthur and Louzis, 1988).

Scientifically, the most interesting aspect of these introductions of myxoma virus in the field was to determine the evolutionary changes that might happen to the virus and to its host. This problem was mainly investigated in Australia and England. A complete discussion is beyond the scope of this review, so only a general outline will be given. In both countries, the trend was the same. On one hand, the viral strains of very high virulence were replaced by strains of intermediate virulence, even though more attenuated strains were sometimes recovered from the field. On the other hand, selection of resistant rabbits was stimulated by the emergence of attenuated viral strains that allowed the survival of moderately resistant animals (Kerr and Best, 1998).

Studies on the co-evolution between the myxoma virus and rabbits are still in progress and recent results seem to indicate that the selection of more virulent viral strains is now in progress because resistant rabbits are less effective transmitters of the virus (Kerr and Best, 1998).

Interestingly, apart from these changes of virulence, a new form of the disease (amyxomatous myxomatosis) has also emerged. The clinical signs of amyxomatous myxomatosis are mainly respiratory, skin nodules being few and small. It might be thought that amyxomatous myxomatosis would not spread via vectors but through direct contact, and would arise predominantly in intensive enclosed rabbitries. However, this last opinion must be viewed with caution since the disease has also been observed in wild rabbits. So far, these forms of myxomatosis have been reported only in France (Brun *et al.*, 1981b; Joubert *et al.*, 1982), Spain (Rosell *et al.*, 1984) and more recently in Belgium (Marlier and Vindevogel, 1996).

1. Aetiology

Myxoma virus (MV), the agent responsible for myxomatosis, is a member of the *Poxviridae* family, which are amongst the largest animal viruses. It has

been designated by the International Commitee on Taxonomy of Viruses as the type species of the *Leporipoxvirus* genus (Francki *et al.*, 1991). Like many other poxviruses, the myxoma virus has a very narrow host range, and is preferentially transmitted mechanically by biting arthropods. By electron microscopy, the virions of myxoma virus are indistinguishable from those of the prototype poxvirus, vaccinia virus. Myxoma virus has a double-stranded DNA of 161 kbp, with a central part containing highly conserved enzymatic and structural genes required for maintenance of the normal viral life cycle. Peripheral regions of the DNA, within and near the inverted terminal repeats (ITR) at both sides of the genome, encode non-essential factors that contribute to the modulation of the host response to infection, leading to the pathogenicity and tropism of the virus (Moss, 2001).

We still know little about the fundamental mechanisms that mediate the host tropism of individual poxviruses and at least three levels of tropism can be defined (Mc Fadden, 2005). Firstly, an evident requirement for a virus to be pathogenic is for it to penetrate and multiply within target cells. This cellular tropism is defined by the virus replication in permissive, semi-permissive or abortive cultured cells of different lineages or species. In general, tropism specificity in cultured cells is mainly determined by specific receptors for viruses, but, for poxviruses, no specific host-cell receptors have ever been identified. Nevertheless, some of the factors, called host-range genes, responsible for host restriction *in vitro* were identified. An ankyrin-repeat host-range protein, the M-T5 protein, was thus shown to be necessary for the myxoma virus replication in rabbit T lymphocytes (Mossman *et al.*, 1996). Its role remains to be determined, the M-T5 host-cell targets being still unidentified.

The two other levels of tropism, tropism for tissue and organism, refer to *in vivo* mechanisms which are not well-known. Most probably, they are mainly linked to the adaptation abilities of the virus to the host immune and inflammatory responses. Indeed, in order to survive in the antagonistic environment created by the host, the myxoma virus, like other poxviruses, has developed strategies to multiply more efficiently and to fight against the host's immune response more specifically. This has led to the emergence of proteins acting as "potentializers" of the viral multiplication, viral factors facilitating or stimulating replication within the host's cells. Their lack results in a decreased infectivity *in vitro* or in a lesser pathogenicity *in vivo*, both traits being measured by comparison with the wild parental strain. Among these potentializers are thymidine kinase protein (Jackson and Bults, 1992) and also viral growth factor of the EGF (Epidermal Growth Factor) (Upton *et al.*, 1987; Opgenorth *et al.*, 1992) type, and EEV-form envelope proteins that facilitate virion diffusion (Smith, 1993).

Other proteins are considered to be modulators of the host's response. The specific and non-specific defence mechanisms used by infected individuals have given rise to original responses on the part of poxviruses. More often than not, they comprise escape mechanisms in the form of decoys**:** viral proteins able to mimic cytokines and thus termed "virokines" (Johnston and Mc Fadden, 2003). Occasionally, the virus produces proteins (termed "viroceptors" because they play the role of pseudo receptor) which trap intercellular messengers (Johnston and Mc Fadden, 2003). They can inhibit the action of certain cytokines. Thus, MT-2 (Upton *et al.*, 1991) and M-T7 (Upton *et al.*, 1992) MV proteins are homologues of cellular receptors of TNF and IFN-γ, respectively. MT-1 is a secreted protein which binds to chemokines (Graham *et al.*, 1997; Lalani *et al.*, 1999). *SERP-1* (Upton *et al.*, 1990), one of the three genes coding for serpins (**ser**ine **p**roteinase **in**hibitors; (Carrell and Travis, 1985)) identified so far on the MV genome, is located in two copies in the ITR and is a secreted factor implicated in the modulation of the inflammatory response (Macen *et al.*, 1993; Nash *et al.*, 1997; Nash *et al.*, 1998).

Moreover, some intracellular viral proteins, termed virotransducers, are able to inhibit innate antiviral pathways. These factors can have a modulating action on the inflammatory response or inhibit apoptosis. Serp2, the second MV serpin, has been identified near the right ITR (Petit *et al.*, 1996) and is closely related to cowpoxvirus-encoded Crm A. It has been shown to be an intracellular inhibitor of caspase-1 and Granzyme B, and to interfere with both inflammation and apoptosis (Messud-Petit *et al.*, 1998; Turner *et al.*, 1999). Serp 3 has an atypical serpin motif and its host-cell targets are still unidentified, but the inactivation of the *Serp 3* ORF led to a significant attenuation *in vivo* (Guerin *et al.*, 2001). The MV M11L protein, an important virulence factor in lymphoid cells, is targeted to the mitochondria (Graham *et al.*, 1992), where it inhibits pro-apoptotic changes in mitochondrial permeability. MNF, an MV ankyrin-repeat protein, is reported to interfere with the NF-kB pathway and to reduce the host inflammatory response (Camus *et al.*, 2004)

Finally, other viral proteins, referred to as virostealth proteins, can act by masking the visible signals associated with virus infection. Among them, MV-LAP downregulates MHC-I molecules and abrogates CTL recognition (Guerin *et al.*, 2002), and also mediates MV-induced CD4 downregulation (Mansouri *et al.*, 2003).

Of course, the current list of factors of pathogenicity is far from being exhaustive and ongoing studies being carried out on various

poxviruses demonstrate their extraordinary capacity to counter the host's defence mechanisms.

2. Clinical signs and pathology

The clinical signs of myxomatosis differ according to the strain of virus, its passage history and its virulence (Fenner, 1994). Two forms of the disease have been identified to date: the nodular (classical) form and the amyxomatous (respiratory) form.

Florid skin lesions and severe immuno-dysfunction, accompanied by supervening Gram-negative bacterial infections of the respiratory tract, characterize the nodular myxomatosis syndrome caused by a virulent myxoma virus strain. Prototype strains of virus deriving from the Australian and European outbreaks have been designed which characterise the various virulence grades (from grade I to grade V) as determined in laboratory rabbits (Fenner and Ratcliffe, 1965).

After infection with a grade I (the most virulent) strain, the first sign of infection is a lump at the site of infection, which increases in size and usually becomes protuberant and ulcerates. An acute blepharo-conjunctivitis and an oedematous swelling of the perineum and scrotum gradually develop. The secondary skin lesions appear on about the sixth or the seventh day (Fenner, 1994). Death usually occurs between the eighth and fifteenth day after infection.

After infection with grade II to V strains the clinical signs are usually the same, with the exception that they evolve more slowly and are less severe. When animals survive, the lesions progressively heal. The mortality rate fluctuates between 100 and 20%, according to the viral strain. The natural mode of transmission of the nodular form is by biting insects. This form is mainly observed in small-scale rabbitries (Arthur and Louzis, 1988).

The clinical signs of amyxomatous myxomatosis are mainly respiratory, skin nodules being few and small. After inoculation of SPF rabbits with five amyxomatous myxoma virus strains (Marlier *et al.*, 1999a), the main clinical observations were the abnormal appearance at the inoculation site and the small number or absence of secondary skin lesions. An acute serous blepharo-conjunctivitis, the intensity of which varied with time and virus strain, was a consistent observation. It began with conjunctival oedema and redness associated with photophobia, and progressed to a thickening of the eyelids that sometimes led to closure of the eyes. Acute respiratory distress was only observed in some animals. When the same five strains were inoculated in conventional rabbits (Marlier *et al.*, 2000c), a much more acute myxomatosis syndrome was reproduced. Both the respiratory and cutaneous expression of the disease was more pronounced in conventional rabbits than in SPF animals. However, the main clinical signs were of the respiratory type. Some animals showed secondary skin lesions, but the skin nodules were of reduced size and never became prominent or exudative. From the comparison of three studies on amyxomatous myxomatosis (Marlier *et al.*, 1999b; 2000a, 2000c), it cannot be concluded that the pneumotropism of amyxomatous myxoma virus strains is greater than that of nodular strains; only the expression of the ectodermotropism is clearly reduced. The development of a more or less severe respiratory distress is due to bacterial superinfections, probably complemented by immunosupression. Therefore, the clinical diagnosis of the amyxomatous form is clearly more difficult than the classical form of myxomatosis .

In nodular forms, the clinical signs are so characteristic that diagnosis can be made on the basis of the clinico-pathological syndrome. In contrast, the diagnosis of attenuated typical myxomatosis or of atypical (amyxomatous) forms most frequently involves the isolation of the virus by inoculation of sensitive cell lines such as the RK-13 cell line (ATCC CCL37) and identification of the virus as myxoma virus by indirect immuno-fluorescence or indirect immunoperoxidase test (Marlier *et al.*, 1999b).

In both cases the definitive diagnosis can be obtained by demonstration of MV nucleic acid by PCR, primers being dedicated to MV essential genes.

3. Epidemiology

There are few studies on myxomatosis in intensive rabbit production units, either on the nodular or the amyxomatous forms.

Deutrich and Hausburg (1986) reported that the mean annual proportion of rabbit farms affected with clinical forms of myxomatosis was 13.5% during the period from1978 to 1980. Rosell *et al.*, (1992) found that the proportion of farms with clinical cases of myxomatosis varied from 13.0 to 22.8% from January 1986 to December 1990. Ghram *et al.* (1996) detected antibody to MV in 54.9% of the rabbitries; no clinical signs being observed in 72% of these infected farms.

In a study of 66 rabbits with no history of vaccination against myxomatosis, which died of pulmonary lesions, myxoma virus was isolated from 10.6% of rabbits and serological evidence of MV infection was demonstrated in 44% of rabbits. No relationship could be established between presence of specific antibodies to MV and the observed pulmonary lesions or the results of bacteriological examinations of lungs (Marlier *et al.*, 2000b).

Marlier *et al.* (2001) have studied the seroprevalence of myxoma virus specific antibodies in 16 farms considered free of myxomatosis on the

basis of the absence of typical clinical signs. MV antibodies were detected by ELISA (sensitivity 100%, specificity 90%) in all 16 farms, the corrected seroprevalences (95% confidence interval) being 55.2 ± 7.73% and 37.0 ± 6.13% for does and broilers respectively. The association between some conditions (herd sizes, types of rabbitries, presence of recurrent respiratory, digestive or reproductive troubles) and seroprevalence of MV antibodies was determined by means of Tarone's univariate chi-square test and by means of logistic regression analyses. In all models, the seroprevalence of MV antibodies was significantly higher in herds (does and broilers) with recurrent respiratory or digestive troubles than in herds without these problems. In broilers, the seroprevalence was higher in herds where animals were housed totally or partially in outdoors rabbitries than in totally enclosed rabbitries. The effect of herd sizes on the presence of MV antibodies was the same in does and broilers, the intermediate sizes being at lower risk than the smaller and larger ones. In does, the univariate analysis shows that seroprevalence is higher in herds with reproductive problems than in herds where they are not present. The logistic regression model could not confirm this finding because of the confusing effect of herd sizes.

4. Prophylaxis and control of myxomatosis

Control of myxomatosis is difficult because of its epidemiological characteristics. Indeed, nowadays, it is not feasible to control the disease in wild rabbit populations. Myxomatosis can only be controlled in domestic and commercial rabbit colonies by a combination of physical measures and vaccination.

In rabbitries it must be emphasised that myxomatosis is potentially sexually transmissible for long periods. Castellini *et al.*, (1994) demonstrated infectious MV in the sperm of inoculated male rabbits for as long as 42 days post infection (dpi). Females inseminated with the contaminated semen became pregnant but died of myxomatosis before parturition. Foutain *et al.*, (1997) studied the replication of MV in the testis of rabbits inoculated with the highly attenuated MV strain Uriarra/2-53/1. They could not detect the virus by plaque assay of homogenized testis tissue later than 20 dpi, but viral DNA was demonstrated by the polymerase chain reaction up to 120 dpi. Finally, Marlier *et al.;* (2000c) succeeded in isolating infectious viruses from the testes of three of six previously inoculated male rabbits for as long as 108 to 125 dpi.

The fact that myxomatosis is potentially sexually transmissible for long periods may seem surprising. However, it helps to explain the introduction of the disease in intensive enclosed rabbitries either in a certain area or during a period with few biting insects (mosquitoes or fleas). Testing of semen before diffusion for artificial insemination should also be promoted.

There is no specific treatment for myxomatosis. For pet rabbits, maintaining affected animals above 30 °C is sometimes recommended since it has been demonstrated that for attenuated strains mortality rates decrease considerably between 20 and 30°C (Fenner and Ratcliffe, 1965).

4.1. Physical measures

Producers of domestic rabbits who live in countries where myxomatosis occurs in wild rabbits may need to protect their stocks from infection even though physical measures alone are far from being fully effective to prevent MV infections in a flock. First, mosquito proofing of animal quarters is recommended, and the health status of new animals should be tested (otherwise, rabbits should be quarantined). Colonies with the disease should be sacrificed, the premises controlled for insects and disinfected. It must also be remembered that veterinarians must deal with this infection in accordance with the laws of their own country. One must also bear in mind that myxomatosis is potentially sexually transmissible for long periods and that consequently trade of semen and/or introduction of new animals could play a role in MV infections.

4.2. Vaccination

Vaccination is very efficient in reducing the spread of myxomatosis, especially for breeding animals. This is currently achieved by using heterologous vaccines based on Shope fibroma virus (SFV), another *Leporipoxvirus*, or homologous vaccines based on cell culture attenuated strains of myxoma virus. In France, primary vaccination is done with the Shope fibroma virus, and a cell culture attenuated strain of myxoma virus, named SG33, is used for booster injections (Saurat *et al.*, 1978). Nevertheless, it was observed that some attenuated strains of myxoma virus could show residual pathogenicity for young rabbits (Brun *et al.*, 1981a).

In an experiment designed to compare two vaccination schemes for their ability to protect rabbits against a challenge with either a virulent amyxomatous MV strain or a virulent nodular MV strain, vaccination with SFV alone failed to prevent clinical signs, naso-conjunctival shedding or tissue infection. Vaccination with SFV followed by a booster inoculation with SG33 protected rabbits against the development of clinical signs and significantly reduced both viral shedding in naso-conjunctival exudates and viral infection of eyelids, lungs and testes; the virus was, however, isolated from the testes of some surviving animals (Marlier *et al.*, 2000a).

5. Myxoma virus and Biotechnology

Various micro-organisms (such as bacteria, viruses, yeasts) are capable of accepting foreign genes, expressing them and consequently, inducing good immunity after administration to animals (Rabinovich *et al.*, 1994). Among the numerous candidate viruses for this role of vector, the best known and the most common system is one based on the use of recombinant infectious poxviruses, particularly the Vaccinia virus (Mahr and Payne, 1992). Other poxviruses, including MV, were later used as vaccine vectors, generally with the same level of success, (Boursnell, 1992), (Robinson and Little, 1992). MV was thus used as a vector for vaccination or immunocontraception.

The first example of recombinant vaccine based on MV was the SG33-VP60 viruses, which protect rabbits against myxomatosis and RVHD simultaneously, either by intradermal or oral administrations (Bertagnoli *et al.*, 1996). Then other recombinant myxoma-RHDV viruses based on a naturally attenuated field strain of myxoma virus have been shown to confer horizontal transmissible protection either by direct contact or in a flea-mediated process (Bàrcena *et al.*, 2000). These results give rise to the possibility of wild rabbit vaccination. Recently, the potential of myxoma virus as a vector for vaccination of other animal species was demonstrated (McCabe *et al.*, 2002)

Finally, the old idea of using myxoma virus as a biological control agent in Australia was updated in the 90s. The Australian Vertebrate Biocontrol Center (VBC) proposed a new method for controlling rabbit populations: the development of an immunocontraceptive vaccine (Robinson and Holland, 1995). The choice of this strategy was based on the concept that fertility control, i.e. limiting the reproductive capacity of a species, is the best means of long term population control. The genes coding for various gametes proteins were inserted in the genome of virulent myxoma virus to be expressed during the replication of the virus. The surviving rabbits would then develop an immune response not only to the virus but also to the protein product of the inserted genes, rendering them infertile (Holland and Jackson, 1994). However, different points (choice of antigens, protocols…) remain to be clarified in order to consider field applications.

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3.3. Pasteurellosis in rabbits

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Introduction

Pasteurellosis is one of the most common bacterial diseases in rabbits, in commercial breeding farms as well as in laboratory facilities. It is mainly characterised by disease of the respiratory system and often by pyogenic affections of other organs, and even septicaemia.

Pasteurellosis has been known for a long time (Davaine, 1872). The germ was thoroughly described by Smith (1887) under the name *Bacterium lepisepticum* and classified in the genus *Pasteurella* by Lignières (1898, 1901). At the current stage of knowledge, three types of pasteurellosis can be considered:

- pasteurellosis in older rabbits (pet rabbits, rabbits used for long-term experiments, serum-producing rabbits).

- pasteurellosis in rabbits kept in small-scale breeding farms, where the level of sanitary prophylaxis is low, disinfection difficult or rare and the level of technicality average or poor.

- pasteurellosis in modern, intensive and industrial-like breeding farms.

The literature addressing the last two types is rather recent, scarce and originates primarily from continental European countries where rabbit production is spread or increasing (Italy, France, Spain, Hungary, Belgium, etc.).

The literature is profuse and mainly American or English for the first type, as shown by Flatt's (1974) and above all Manning's (Manning *et al.*, 1989) reviews. The latter is particularly instructive; yet, many parts can hardly or cannot be transposed to pasteurelloses in industrial rabbit farms.

Rabbit pasteurellosis is a very important disease, which is demonstrated by the number of books on rabbit pathology addressing this disease (Lesbouyries, 1963; Kötche and Gottschalk, 1983; Marcato and Rosmini, 1986; Lebas *et al.*, 1986; Vetési, 1990; Boucher and Nouaille, 1996).

It is of economic concern (Rosell *et al.*, 1992a, 1992b) in rabbit production and is responsible for at least 50% of the major causes of breeding doe culling (Balençon *et al.*, 1982). From a medical standpoint, it is a severe disease because means of control are complex and treatments expensive, lengthy and often poorly effective. It is also epidemiologically severe for many rabbit breeding farms because infectious carrier rabbits are highly widespread (Vetési, 1990). Finally, pasteurellosis, which can be transmitted from one animal species to another, is a minor zoonosis (Avril and Donnio, 1987).

1. Epidemiology

Although knowledge on rabbits dates back many years, true domestic keeping of this animal only actually started in the 19th. century. It is noted that the cradle of the rabbit (*Oryctolagus cuniculus*) is the Iberian peninsula (Rougeot, 1981; Camps, 1995). The Roman armies took it across the Pyrenees and the great explorers in the Middle Ages spread it throughout the rest of the world. Needless to say, along with its distribution went the transportation of its diseases. Therefore, rabbit pasteurellosis occurs wherever the animal lives. It is present on all the continents.

For the last few years, the diffusion of certain *Pasteurella* strains throughout European rabbit-producing countries occurred via trade networks. In

a given region and over the same period of time, various and identical forms of the disease can be found; however, there are no large-scale regional epidemiological phenomena.

As infectious carriage widely prevails (Lebas *et al.*, 1986; Rosell *et al.*, 1992a, 1992b), an outbreak in a rabbitry will depend on contributing or triggering, environmental or physiological factors. Then, as the effect of these factors tapers off or disappears, remission of disease can be observed to a certain extent, later followed by its reappearance under the same or different form. If left unattended, the disease can take on severe enzootic characteristics, the evolution of which no longer depends on environmental factors. Besides, in countries with very low relative humidity due to very low winter temperatures (Canada, Poland), pasteurellosis rages endemically during this season when large numbers of rabbits are concentrated indoors.

This diversity of descriptive epidemiology accounts for the abundance of clinical manifestations and perhaps their constancy through the decades.

1.1. Infection

Infectious, chronic carrier rabbits themselves represents the main source of infection. Breeders are constant *Pasteurella* reservoirs within the breeding colony itself (Coudert *et al.*, 1986). However, infection by new strains may result from the introduction of rabbits in a new rabbitry or the addition of rabbits into an existing colony following stock culling. An enzootic rabbit pasteurellosis with high mortality caused by pyometra in breeding females and by pyothorax in their progenies was reported recently (Virag *et al.*, 2004). This episode emerged within the quarantine period following the breeding stock’s relocation, while the original rabbits of the same farm did not show signs of pasteurellosis.

Besides, insemination material may be an important vector of vaginal infection. Given the anatomical morphology of this organ, the insemination pipette may introduce the germ deeply and the mucous membrane may be damaged. Depending on the insemination instrument used, the level of hygiene observed by the operator and his experience, it is estimated that 30% and 60% of insemination failures may be due, in certain breeding stocks, to vaginitis or even metritis induced by insemination itself or previous gestations (from 6 to 9 per year!). In these cases, the germs frequently isolated are *Pasteurella* bacteria.

Natural mating also could spread the bacteria interestingly from the nasal mucosa of the serving buck to the vaginal mucosa of the doe (Virag *et al.*, 2004).

Nasal and mammary secretions and abscess content represent the primary source of the virulent material which could be taken up by direct contact. Secondly, there are objects and equipment that are directly contaminated by secretions or pus. *Pasteurella* do not survive a long time outside the organism. Airborne transmission, in the strict sense of the term, plays a minor role (Digiacomo *et al.*, 1989; Rideaud and Coudert, 1992b), except when the air is loaded with dust. The nasal route is the major route of penetration. The conjunctival, oral, transcutaneous and vaginal routes have also been described.

1.2. Receptivity

**** Extrinsic factors***

Rabbit *Pasteurella*, aside from some virulent strains, are facultative pathogens. Extrinsic factors play an important role in disease outbreak. The rabbit upper respiratory tract constitutes a very effective means of defense. However, the structure of rabbit sinuses is highly complex and the mucous membrane is very fragile. Thus, rapid air velocity or too low relative humidity will result in drying-up of the mucous membrane and increased sensitivity of the epithelium to infection. An ammonia level above 5 ppm is enough to paralyse the epithelial cilia in rabbits and promote rhinitis. Pelleted feeds containing too many "fines" (poorly agglomerated particles) will have the same effect. The poor hygiene of the rabbit's immediate environment (cages, drinking troughs, hoppers) as well as overcrowding are promoting factors. Dirty floors (cage bottoms) keep endemic mastitis alive.

Handling of breeding does is very frequent in professional breeding, particularly for abdominal palpation (diagnosis of gestation), which is performed in series in the maternity or doe herd and represents a factor of diffusion of mastitis in a colony. The risk is even greater if palpation occurs when does have reached a peak of lactation; i. e., when the udder is highly functional. During the constitution of a new colony or after depopulation, pasteurellosis may appear a few weeks later while the original breeding colony shows no clinical signs; the mere environmental change is one of the factors contributing to disease outbreak. Following colony restocking, if animals no longer have the same origin, new strains of *Pasteurella* may be introduced and trigger severe endemic pasteurellosis.

**** Intrinsic factors***

Mortality due to pasteurellosis might be less frequent in rabbits of the New Zealand White breed than the Flemish Giant breed (Dillehay *et al.*, 1991). However, with this type of data, it is difficult to dissociate genetic factors from environmental factors. Nevertheless, in a constant environment, Coudert and Brun (1989) also found resistance differences between rabbit breeds: the Californian breed seems to be more sensitive to pasteurellosis than Californian x New Zealand White-crossed

breeds, the latter being itself more susceptible than purebred New Zealand rabbits.

Sex is not involved. The apparently higher number of affected females is proportional to the absolute number of females in the maternity and to the frequent handling that they undergo.

Age is a very important factor. The "natural" resistance of young rabbits before weaning has been demonstrated (Rideaud and Coudert, 1992b). Indeed, under good general environmental conditions, young rabbits remain *Pasteurella*-free until 21-25 days of age, even if the dam is a healthy carrier. This does not merely result from passive immunity transmitted through maternal milk as experimental inoculation trials on young rabbits born from SPF dams have turned out unsuccessful (Rideaud *et al.*, 1998). Yet, natural contamination of young rabbits under the dam may occur in farms where pasteurellosis is endemic (Patton *et al.*, 1984; Digiacomo, 1992). In a clinical symptom-free breeding farm, Rideaud *et al.*, (1992a) described the evolution of *P. multocida* asymptomatic carriage in the middle ear and found it to be 0% at 4 weeks of age, 3% at 8 weeks, 12% at 11 weeks, 18% at 14 weeks and 50% after the first parturitions.

The physiological status of the female may be a factor determining *Pasteurella* infection receptivity (Coudert and Brun, 1989). In semi-intensive and intensive breeding systems, the producer too often considers the breeding doe as an "automatic distributor" of young rabbits and forgets that it is also a biological being which will achieve 6 or 7 gestations and lactations during the year, or even more! By the end of gestation, as in any mammal, it is characterised by profound physiological changes and an increased frailty partly due to a few days of immunodepression. It is in the few days preceding parturition that all the pre-existing pathological phenomena, particularly respiratory diseases, will be expressed.

Besides, as time goes by, the doe's uterus is put through the mill and after the 8th or 9th litter, chronic *P. multocida* carriage is to be expected, which is associated with middle-ear carriage. Bucks are also a significant source of infectious carriers as producers frequently keep them as long as possible, provided they are very active during mating and remain fertile.

2. Symptoms

With the development of modern, commercial rabbit production, many diseases have waned and even disappeared while others have emerged. This is not the case for pasteurellosis, which has remained constant in time. Since Webster's works in (1924a, 1924b, 1924c), few new findings have been reported about the various forms of pasteurellosis.

2.1. Respiratory form

It is the most frequently described form of the disease, perhaps because it is the most visible. Symptoms are first localised in the upper respiratory tract (rhinitis, tracheitis). Hair of the nose and forepaws is wet and soiled by nasal discharge. The animal sneezes often, thereby expelling serous or mucopurulent material in the air. This acute rhinitis can evolve toward the chronic stage. In the final stage, part of or the whole nasal turbinates waste away or disappear (Kpodekon, 1983a). This phenomenon drove investigators to think that an analogy could exist with swine atrophic rhinitis (Digiacomo, 1989; Frymus *et al.*, 1991). Although the same germ is involved in both cases, strains and pathogenesis are not identical. Pneumonia and pleuropneumonia are the ultimate outcomes of the respiratory form of pasteurellosis. They are characterised by hyperthermia and dyspnoea, which rapidly leads to death. At this stage, any coryza symptom may have disappeared.

This respiratory symptomatology is not very different from that observed with other bacteria, such as *Staphylococcus*, *Streptococcus* and *Bordetella*.

2.2. Septicaemic form

The first biological endeavour to eliminate rabbits was carried out on a large estate in the late 19th century using *Bacterium lepisepticum* (i. e., *P. multocida)*. It was reported that all rabbits died in a few days (Webster, 1924a). Septicaemia primarily occurs in small-scale commercial farms where rabbits and poultry are kept together. Cases have been reported of natural contamination of rabbits by *P. multocida* strains originating from hens; the rabbits, which had been placed in a former aviary, died of septicaemia in less than 24 hours. This has been experimentally confirmed; indeed, the most septicaemic strains in rabbits often come from poultry (Coudert, 1997, unpublished work).

However, septicaemia is rare or seldom diagnosed. Indeed, in overacute cases, the animal can die without any apparent symptoms (Lesbouyries, 1963). Sometimes, it retires in a corner and is listless, huddled down; hyperthermia can reach 41°C. Death occurs 24 to 48 hours following the onset of symptoms. Most often, no respiratory signs are observed. Septicaemia can develop into other clinical forms: rhinitis, pleuropneumonia, abscesses, etc.

2.3. Abscesses

The rabbit is known for being liable to develop abscesses (*Streptococci*, *Staphylococci*, *Pasteurella*, etc.). Overall, infections with abscesses represent the major cause of breeding doe elimination (Coudert, 1980). In small-scale breeding, staphylococcal abscesses prevail while in commercial breeding,

Pasteurella abscesses have become the most frequently encountered. The main reason for staphylococcal abscess regression is the improved hygienic status necessarily resulting from rabbitry modernisation; nevertheless, trade networks have spread pathogenic staphylococcus strain-carrier rabbit breeds on a large scale. The development of trade of selected commercial meat rabbit breeds also contributed to the rapid diffusion of the same *Pasteurella* strains (pathogenic or not) throughout Europe. In a few years' time, the major abscess aetiology could thus be modified.

The size of *Pasteurella* abscesses ranges from that of millet to that of a lemon. They may be sometimes very bulky and cumbersome, but the rabbit may not necessarily appear much affected. They develop in various sites; they may be subcutaneous, subserosal, submucosal, retrobulbar, genital, seldom plantar, etc. Localisation in the middle-ear cavity results, more often than not, in otitis media supurrativa (OMS) chronica, which is of particular interest.

2.4. Otitis and encephalitis

In rabbitries, over 60% breeding does have *Pasteurella* OMS possibly associated with other forms of pasteurellosis (Coudert *et al.*, 1986). Most of the time, this OMS is asymptomatic. It can develop into otitis interna and/or encephalitis. In this case, animals, particularly the young, show symptoms of nervous disorders: torticollis, vestibular disorders (Khera *et al.*, 1971). The head generally tilts towards the affected side.

The frequency of this middle-ear localisation is certainly the most interesting epidemiological factor to be considered for it constitutes a real *Pasteurella* reservoir. More than that, it is a cavity, and once pus has formed in it, it is well known that germs are safe from even the most radical antibiotic treatments. This explains relapses, which almost invariably occur after treatments.

2.5. Metritis and mastitis

Although the existence of *Pasteurella* metritis and vaginitis are known (Flatt, 1974; Johnson and Wolf, 1993), these are not frequent. Their frequency increased with rabbit production intensification in Europe. The popularisation of artificial insemination methods led the pathology to soar, sometimes dramatically in certain rabbitries when implementation of this method failed to meet professional sanitary standards. Metritis is a suppurative pathology and is often characterised by a pyometra. Generally, pregnant females do not abort, but an embryonic lysis occurs. These affections result in infertility or death for does after intoxination or septicopyohemia. As for *Pasteurella* mastitis, it overtakes mastitis of staphylococcal origin, as has been reported for abscesses.

2.6. Other localisations

Numerous other forms of pasteurellosis have been described, such as mandibular osteomyelitis (Hinton, 1978), peritonitis (Bjotvedt *et al.*, 1979), tibiotarsal osteoarthritis (Hago *et al.*, 1987) and dacryocystitis (Petersen Jones and Carrington, 1988). These forms of the disease are often isolated cases and almost inexistent in intensive production.

3. Lesions

3.1. Gross lesions

**** Respiratory form***

The nasal cavities contain pus or mucopus. The mucous membranes are highly congested. Sometimes, the nasal turbinates have partially or totally disappeared, in which case the cavity contains pus mixed with blood. Opening of the thoracic cavity reveals interstitial pneumonia and/or suppurative bronchopneumonia, which are often associated with unilateral or bilateral fibrinous pleurisy and sometimes fibrinous pericarditis.

**** Septicaemic form***

Autopsy of the animals reveals serous-catharral rhinitis, serous conjunctivitis and multiple subserosal haemorrhages (mainly pleura and pericardium). Septicaemia may be accompanied by other organic lesions. The liver is congested, but the spleen generally looks normal.

**** Otitis and encephalitis***

Opening of the tympanic bulla reveals a middle ear filled with a serous-purulent exudate in acute cases (Fox *et al.*, 1971; Kpodekon, 1983b). The mucous membrane is hyperaemic, congested and often oedematous. In chronic cases, it is thickened; the tympanic cavity contains thick yellowish-white pus. The asymptomatic presence of *P. multocida* in the brain is almost as frequent as in the middle ear (Balençon *et al.*, 1982). However, the brain may be the site of sometimes bulky abscesses; consequently, meningeal congestion and serous meningitis are often encountered (Kpodekon, 1983a, 1983b).

**** Metritis and mastitis***

Opening of the uterus discloses whitish pus and a congested and thickened endometrium. Metritis is often accompanied with vaginitis, salpingitis and peritonitis. The mammary glands are the site of a parenchymous inflammation which is susceptible to develop and form abscesses.

3.2. Microscopy

Diseased organs are infiltrated by inflammatory cells, primarily granulocytes, along with a few lymphocytes and plasmocytes, a picture characteristic of a suppurative inflammation (Redondo *et al.*, 1993). Abscesses have a usual microscopic structure. In subacute or chronic otitis

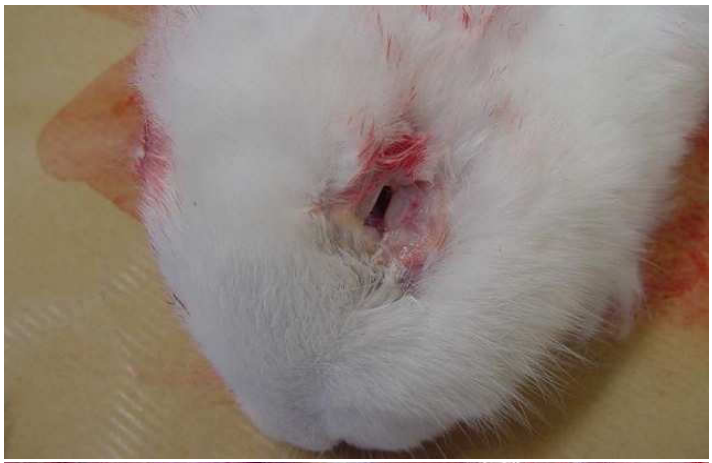

Figure 1. ***P****urulent conjunctivitis: young rabbit before weaning, unilateral conjunctivitis* (Courtesy of B. Le Normand).

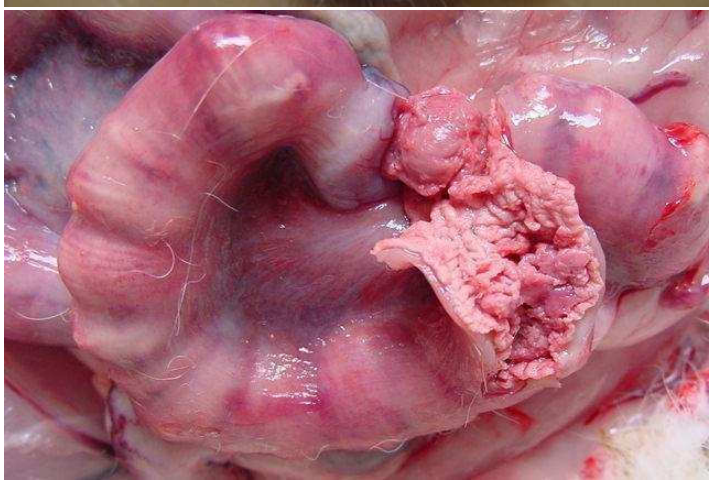

Figure 2. *Caseous metritis and momified fetus: female, problem of infertility on 1 breeding* (Courtesy of B. Le Normand).

Figure 3. *Internal otitis: female* (Courtesy of B. Le Normand).

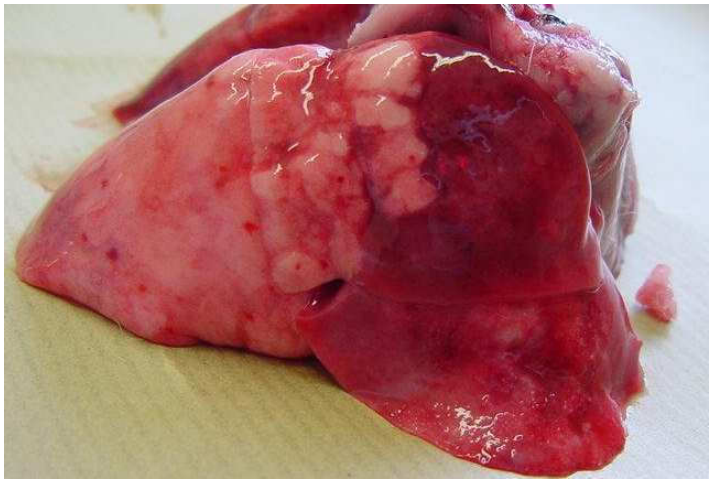

Figure 4. *Red pneumonia cranial lobes: fattening rabbits, traditional lesions of pasteurellic pneumonia* (Courtesy of B. Le Normand).

media, epithelial metaplasia may be observed on the mucous membrane. As a consequence, the simple epithelium is replaced by a malpighian-type epithelium, keratinised or not (Flatt *et al.*, 1977).

Overall, rabbit pasteurellosis can be described as a disease evolving rather chronically and the symptoms of which, although showing great diversity, take on the form of only one type of anatomical lesion: suppurative inflammation. What are the factors behind this uniformity of lesions?

4. Aetiology

4.1. General points

When speaking of rabbit pasteurellosis, the most unique aetiological agent involved is *Pasteurella multocida*. It is an immobile and unsporulated coccobacillus with a diameter ranging from 0.2 and 0.3 micrometers and a length from 1 and 2 micrometers. Pasteurellae are Gram- bacteria (gram negative), recent isolates often with bipolar-staining properties. They can also be stained with methylene blue and fuchsine. They are facultative anaerobic. They are highly sensitive to physicochemical treatments; for example, they can be destroyed in a few minutes at a temperature of 60°C and in 1 or 2 days in a dry environment; they can only resist for a few days at 4°C, but are capable of surviving for weeks in slurries and inside corpses. They are highly sensitive to common detergents and antibodies used against Gram- bacteria.

Pasteurella classification was continually changing until the last few years (Escande, 1985; Mollaret, 1986). The term "*Pasteurella*" was coined as a tribute to Louis Pasteur, following Lignières' works (1901), which enabled the genus *Pasteurella* to be individualised. In 1901, Lignières established a first classification according to the host species, but there was much confusion between pasteurelloses and their aetiological agents. This confusion ceased with the work of Chamberland and Jouan (1906), which enabled the *Pasteurella* genus to be confirmed as a common germ found in the intestine and respiratory tract mucosa of healthy animals. In 1939, Rosenbusch and Merchant proposed the name *Pasteurella multocida* (i. e., killing many species) on the basis of the identity of the biochemical features whatever the host species, animal or human. Only in 1981 was *Pasteurella* permanently introduced into the Pasteurellaceae family with Pohl (1981), and then in 1989 with Mannheim. Mutters *et al.* (1985) and Avril and Donnio (1987) clarified the *Pasteurella* genus taxonomy by proposing subspecies of *Pasteurella multocida*.

Pasteurella bacteriology is not simple and mistaken identification is common. There is no specific culture medium for *P. multocida*. The bacterium grows rapidly, in 18-24 hours, on blood agar or tryptose agar supplemented with 5% horse serum, but not on MacConkey agar unlike actinobacilli, enterobacteria and *Bordetella bronchiseptica*. It is relatively hard to preserve; it must neither be maintained at 4°C, nor in agar Petri dishes as it dies rapidly under these conditions. In order to keep their cultural, biochemical and antigenic characteristics, the strains recovered from agar Petri dishes must be amended in deep agar tube to store bacterial strains (Sanofi, Diagnostic Pasteur reference 63683). This "stock" culture agar also constitutes a good medium for strain transportation or dispatching from one laboratory to another. Cultured bacteria must be kept away from light and at laboratory room temperature, and should not be stored for over a month. Beyond this time limit, bacteria must be kept in more complex culture media, at –20°C or even –70°C. The lack of an adequate culture medium is often a limiting factor for transportation or dispatching of *P. multocida* samples or cultures from one laboratory to another. Kawamoto *et al.* (1951) recommend Cary-Blair or Leibovitz-15 culture media for safely dispatching rabbit nasal samples within a period of 4 days. The advantage of these culture media is that they provide *Pasteurella* bacteria with a survival time superior to 14 days at room temperature. According to Shimoda *et al.* (1991), transportation should be carried out in PBS (pH: 7.0) at 4°C within a period of 8 days. Under this last condition, however, *Pasteurella* survival time dramatically drops unless skimmed milk is added to the medium.

P. multocida strains quickly deteriorate on the agar plate. This dissociation accelerates after several subcultures (Londons and Yaw, 1957) and at the last stage of degradation (rough), the bacterium loses its capsule.

Like most Gram- bacteria, the capsule is made of sugars (Pyliotis and Mukkur, 1981; Manning, 1984). In some serotypes of *P. multocida* isolated from the rabbit, this capsule contains a high amount of hyaluronic acid and, in particular, this amount is higher in capsular serotype A than in capsular serotype D. Hyaluronic acid acts as an adhesive and its effect is all the more significant when the hyaluronic acid level is high (Glorioso *et al.*, 1982; Esslinger *et al.*, 1994). Yet, this capsular component is non-antigenic and makes it impossible for antibodies to be produced (Carter, 1955; Namioka and Murata, 1961). Some strains, the more virulent ones, bear micropili (fimbriae) all around, which also allows them to adhere to the mucous membrane (Glorioso *et al.*, 1982; Botcher *et al.*, 1990. Others produce a dermonecrotizing toxin (serotype D).

4.2. Characterisation of strains

P. multocida serotyping has been done for many years on the basis of an agglutination test (Little and Lyon, 1943) or a mouse passive protection test (Roberts, 1947). Carter (1955) was able to develop an indirect hemagglutination test and classify the strains according to their "capsular antigens" into 4

serogroups: A, B, D and E. Serogroup F, a new capsule serogroup of *Pasteurella multocida* was described later (Rimler and Rhoades, 1987) and these strains were first isolated from turkeys throughout USA. It is remarkable however, that conventional capsular typing methods were not entirely accessible for most laboratories and this resulted a proportion of non-typable strains in numerous studies. Numerous studies (Perreau *et al.*, 1962; Lu *et al.*, 1983, 1978; Chengappa *et al.*, 1982; Jaglic *et al.*, 2004; Manning, 1984; Rideaud and Coudert 1994; Rideaud *et al.*, 1992a) addressed the serological features of rabbit isolates of *Pasteurella multocida*. Serogroups A, D, B and F have been found amongst them. Serogroup A is by far the most widely encountered (Chengappa *et al.*, 1982; Perreau *et al.*, 1962; Rideaud *et al.*, 1992a; Rideaud and Coudert 1994; Lu *et al.*, 1983); serogroup D is rather rarely found (Manning, 1984; Virag *et al.*, 2005; Jaglic *et al.*, 2004) almost exclusively in small-scale breeding farms or laboratory facilities. Serogroup F strains have been shown only at recent rabbit isolates when molecular genetic typing of capsule genes become available (Virag *et al.*, 2005; Jaglic *et al.*, 2004).

In 1961, the Japanese investigators, Namioka and Murata (1961), developed a serotyping method based on "somatic antigens" obtained after treating the germs with HCl and classified *P. multocida* into 11 "somatic" types. The FAO/APHCA seminar-workshop, held in Sri Lanka in 1979, recommended that the combined Namioka-Carter identification be used for determining the *Pasteurella* serotypes responsible for hemorrhagic septicaemias (De Alwis, 1987). However, this method of identification is extremely complex and difficult to implement, particularly in regards to purification of "somatic antigens".

In 1972, Heddleston *et al.* (Heddleston *et al.*, 1972) devised another method of "somatic antigen" precipitation after placing the germs at 100°C for 1 hour. This method permitted them to classify *P. multocida* into 16 serotypes. In 1981, Carter and Chengappa (Carter and Chengappa, 1981) proposed adopting the Carter-Heddleston identification because of the ease of implementation that the Heddleston's test presented. It should be pointed out that no agreement exists between Namioka's and Heddleston's somatic serotypes (Brogden, 1979); for example, Namioka's serotype 5 is different from Heddleston's serotype 5. This results in great confusion in the literature. In France, the method which has been adopted so far is Namioka's. For the rabbit species, identification according to Namioka has proved to be the more discriminating in regards to *Pasteurella*. Indeed, when using Heddleston's method, serotype A:3 is by far the most widely spread in European industrial rearing systems. When using Namioka's method, the serotypes found are of a much larger diversity in the farms in question, which makes it a better tool for pathological or epidemiological studies.

In rabbits, the antigenic characteristics alone have not been sufficient to characterise *Pasteurella* strains according to either their pathogenicity or their epidemiological origin. Strains of the same serotype may have different pathogenicities. In the United States, serotype A:12 (Heddleston's method) could be the most frequent (Chengappa *et al.*, 1982) and the most pathogenic; these results have not been reported in other countries. In regards to *Pasteurella* bacteria found in European intensive breeding farms, it has never been possible to establish a relationship between pathogenicity and serotype (Namioka's method).

Further investigations are necessary and currently focus on the use of biotechnological methods or the kinetic study of biochemical characteristics. The development of DNA-based methods has provided alternative characterisation that overcomes the limitations of phenotyping, while providing differentiation of phenotypically similar *P. multocida* strains. Out of several methods ribo-, RAPD and rep-PCR typing have been used for comparison of *P. multocida* strains isolated from rabbits. Ribotyping in conjuction with REA and RAPD analysis of 41 strains (Chaslus-Dancla *et al.*, 1996) resulted 7-9 and 4-7 genotypes respectively, when combined capsular and somatic serotype differenciated only 4 types. Rep-PCR performed on *P. multocida* strains isolated from nasal and vaginal mucosa of two groups of breeding females resulted in genetic fingerprints where strains clustered apart according to the group sampled. The isolates obtained from the group of shipment-stressed rabbits formed two main clusters according to the site of isolation: nasal or vaginal mucosa (Zaoutis *et al.*, 1991). Analysis of *P. multocida* isolates from the nasal mucosa of healthy rabbits or from pathognomical lesions discovered at autopsy of dead rabbits exhibited genetic heterogeneity but no correlation with the status of the rabbit sampled, suggesting that healthy rabbits may act as a reservoir of pathogenic *P. multocida* strains (Virag *et al.*, 2005). The same technique was performed on strains collected from an intensive rabbitry across time and production level. Genotyping revealed most strikingly more pairs of genetically identical strains, each isolated both from diseased and from healthy rabbits, or one from diseased and another from healthy rabbits. Clustering otherwise did not show obvious association of the genotype with biochemical or serotyping characteristics and pathogenecity of the isolates (Virag *et al.*, 2005).

On the other hand, the analysis of a bacteria's metabolic profile used by Badiola-Saiz *et al.*,(1996) on about a hundred strains, enabled discrimination between effectively pathogenic and mildly or non-pathogenic strains, thereby perhaps paving the way for, at last, efficacious autovaccines.

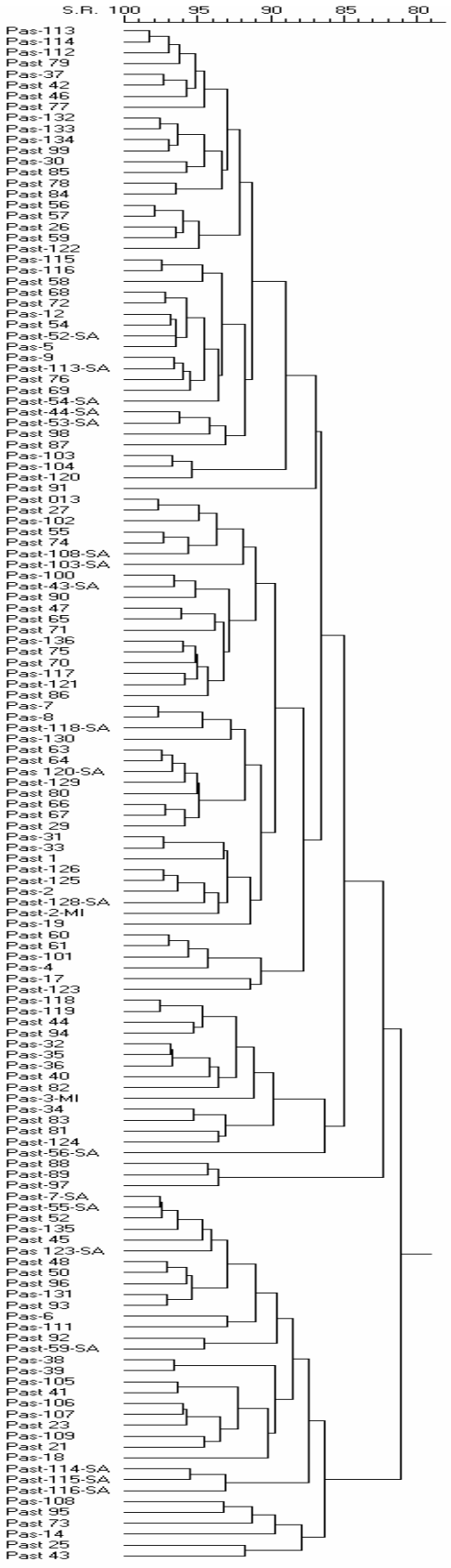

Figure 5. *Dendrogram of the strains isolated in 47 rabbitries – Biochemical kinetics. (Cerrone et al., 2005).*

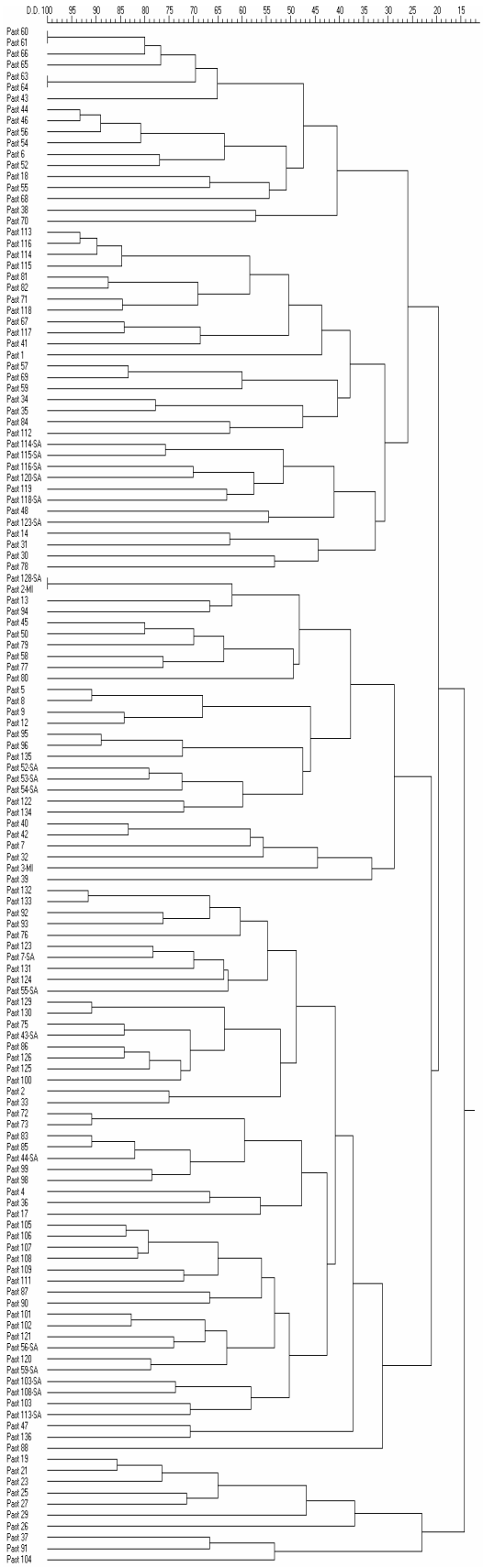

Figure 6. *Dendrogram of the strains isolated in 47 rabbitries – REP-PCR (Band analysis) (Cerrone et al., 2005).*

It is essential to ensure that the relationships of the taxa of genus *Pasteurella* are described more accurately: this should be done with reference to phenotypic and genotypic features so as to address their value in classifying and epidemiological typing of these organisms. The *P. multocida* strains need to be further investigated by using strains from different geographical areas. REP-PCR should be used and the obtained results should be compared with extended phenotyping results.

This technique was used for identifying and typing *Pasteurella multocida* strains isolated from Italian rabbitries (Cerrone *et al.*, 2005) (Figure 5 and 6).

The study refers to the phenotypic analysis (diameter and speed growth of the colonies, biochemical kinetics) and to the genotypic one (REP-PCR) of 141 *P. multocida* strains isolated in 47 rabbitries located in Campania region (Italy). On the basis of the different brake points selected, to separate the different genotypes, the following division was obtained: 14 clusters for Biochemical kinetcs with a RS (Relative Similarity) of 90%, 16 clusters for REP-PCR (Intensity of the Band) with a RS of 65% and 22 for REP-PCR with a DD (Dice Distance) of 45.

It will be of great interest to know if strains coming from different countries will cluster together and if there is/and what is the common link. This molecular epidemiology tool could be also useful to select rabbit lines that are genetically resistant to the disease.

For the past few years, other authors have focused on a complex (morphological, cultural and biochemical) characterisation of the strains (Rideaud *et al.*, 1992a; Rideaud and Coudert, 1994). According to these authors, a positive correlation exists between the diameter of the colonies formed by the various *P. multocida* strains and their pathogenicity in rabbits. Caution however is necessary about generalization, because colony size could be heavily influenced by culture conditions. According to the same authors, the presence or absence of Ornithine DeCarboxylase (ODC) in *P. multocida* is a more reliable discriminating factor. The ODC-free strains, whatever the colony diameter, have been shown to be mildly or non-pathogenic; this is a more reliable criterion for forecasting strain pathogenicities than measuring the colony diameter, for it does not depend on culture conditions. Besides, septicaemic strains all have a large diameter and an ODC. Consequently, pathogenic *Pasteurella* are always ODC+; they form large- or medium-sized colonies.

4.3. Pathogenicity

In general, in *Pasteurella* there is no clear evidence of a relationship between serotype and host species, or the nature of the diseased organ, or the phenotype of the colonies formed in culture media (Rideaud *et al.*, 1992a; Rideaud and Coudert, 1994; Mohan *et al.*, 1994). Rabbit *Pasteurella* virulence varies on a very wide range, which can be divided into 3 categories:

* strains of a septicaemic nature, killing within 24 hours 100% of the rabbits inoculated with less than $1x10^3$ germs placed on the edge of the nares,

* strains killing no rabbits with $1x10^6$ germ-containing inocula,

* between these two extremes, strains killing 20 to 80% rabbits within 2 to 5 days and inducing severe organic lesions.

With regard to lesions, other strains besides septicaemic types can be found, such as significantly pyogenic strains, hemolytic strains and strains with a pronounced hepatic, splenic or pulmonary tropism, etc. These characteristics can combine, but this is not always the case: strains exist which induce severe endemic disease with abscesses, but only mild or even no pulmonary disease.

The animal origin of *P. multocida* strains also seems to play an important role. Strains isolated from poultry (hens, ducks, geese) have proved septicaemic for rabbits (Coudert, 1997 unpublished). This has been confirmed in natural cases of contamination of rabbit breeding farms contaminated by poultry strains. Swine *Pasteurella* are pathogenic for rabbits, yet not septicaemic.

4.4. Antigenicity And Immunogenicity

A high antibody production quickly accompanies *P. multocida* infection. However, the antibody protective potency is very low (Perreau, 1982). Some outer-membrane proteins, liable to be involved in immunity, are specific to rabbit-isolated *Pasteurella multocida* strains (Grossmann *et al.*, 1998). Moreover, the protective potency is specific to the contaminating strain. As a consequence for medical prophylaxis, only autovaccines can be efficacious, although in a very relative way.

4.5. Other *pasteurella* species in rabbits

As in rats, mice and hamsters, *P. pneumotropica* may at times provoke respiratory infections in rabbits, such as subacute pneumonia, rhinitis, sinusitis and otitis media (Vetési, 1990). Thigpen (1978) was also able to isolate *P. aerogenes* from a doe uterus after abortion, a bacterium usually considered as an epiphytic microorganism of the pig intestine, sometimes causing abortions in this species. Similarly, *P. haemolytica* was isolated in rabbits raised along with cattle. The almost single aetiological agent of rabbit pasteurellosis remains *P. multocida*.

As this bacterium is a facultative pathogenic germ, the analytical epidemiology of pasteurellosis will obviously be determined by the infection pressure, but most of all by the extrinsic and intrinsic factors of receptivity.

5. Pathogenesis

From the site of penetration, which is most often the nasal cavity, infection will progress in different ways:

- via the trachea towards the lungs, where alveolar macrophages are the first target cells during the first phases of the lung infection (Esslinger *et al.*, 1994)

- via the nasolacrimal duct towards the conjunctiva and the retrobulbar tissue

- via the Eustachian tube towards the middle ear and the brain

- via the nerves (mainly the trigeminal nerves) and the perineural lymphatic system towards the brain (Kpodekon, 1983a, 1983b)

- via the general lymphatic system (Rideaud *et al.*, 1998) and the blood, thereby causing a septicaemia with or without organic lesions.

Let's also note:

- the transcutaneous route, favoured by microtraumas, which causes more or less diffuse subcutaneous abscesses depending on *Pasteurella* strains. With certain strains, the intradermal route of infection very frequently results in intestinal disease. With the same strains, this intestinal pathology is less frequently found when inoculation occurs via the intranasal route (Rideaud *et al.*, 1998). In addition, several authors (Zaher *et al.*, 1987; Tournut and Camguilhem, 1983; Sokkar *et al.*, 1987) have reported spontaneous cases of enteritis due to *Pasteurella* in young fattening rabbits. These cases of *Pasteurella* enteritis have been experimentally reproduced with certain *P. multocida* strains inoculated via the transcutaneous route (Rideaud *et al.*, 1998).

- the vaginal route, the origin of vaginitis, metritis, salpingitis and peritonitis.

The pathogenesis of *Pasteurella* atrophic rhinitis has drawn the attention of investigators for the past several years. Atrophic rhinitis has been reproduced in rabbits inoculated via the intramuscular or intranasal routes with toxins produced by swine or rabbit *P. multocida* isolates (Frymus *et al.*, 1991; Digiacomo *et al.*, 1993).However, the cytotoxicity of the strains used could only be confirmed in vitro on embryonic cell cultures of bovine turbinates. These heat-labile toxins (dermonecrotising in guinea pigs) are primarily produced by serotype-D *P. multocida* strains, which are very uncommon in rabbits (Suckow *et al.*, 1991).

6. Diagnosis

6.1. Clinical aspect

**** Positive diagnosis***

There are absolutely no pathognomonic symptoms. Most of them can also be observed in infections due to *Staphylococcus* or other pyogenic germs, such as *Klebsiella* (pneumonias). Only bacteriological culture can confirm the aetiology as being *Pasteurella*. In practice, particularly in European rabbitries, *Pasteurella* is the most frequent cause.

- *Respiratory form*: coryza is easy to diagnose. Repeated sneezing can easily be heard when entering a rabbitry. The humid and soiled condition of the nares, nose hair and forepaws draws attention. In breeders, dyspnea, sniffing or snoring can also be noted while coryza is absent.

- *Septicaemic form*: the overacute form will not be noticed as in most cases the animal dies without any forewarning signs. In the acute form, hyperthermia and polypnea occur, the animal huddles up in a corner; the diagnosis is difficult.

- *Abcesses*: these can be readily detected on inspection and/or palpation in cases of subcutaneous, retrobulbar and mammary localisation.

- *Otitis media and interna and encephalitis*: are seldom diagnosed, except when a torticollis or a symptom of nervous disorder is present.

- *Metritis and vaginitis*: these are rarely diagnosed on the basis of a mere clinical examination.

**** Differential diagnosis***

- *Viral Haemorrhagic Disease* (VHD): it differs from overacute pasteurellosis in that it strikes more violently and affects a much greater number of rabbits while generally sparing the young under 6-7 weeks of age. Antibiotherapy has no effect on VHD, and most of all this is an epizootic disease; it seldom affects only one isolated rabbitry.

- *Torticollis* and nervous symptoms: in laboratory facilities, in small-scale breeding farms, but most of all in pet rabbits, ear mange complications and granulomatosous encephalitis (*Encephalitozoon cuniculi*) should be considered first. Torticollis is commonly due to this microsporidium (Kunstyr *et al.*, 1986) in pet rabbits, but seldom occurs in commercial or laboratory rabbits (Kunstyr and Naumann, 1985).

- *Pneumocystosis*: very transient interstitial pneumonia caused by *Pneumocystis carinii* has been described around 28 days of age (Dei-Cas *et al.*, 1990; Ceré *et al.*, 1997). The diagnosis can only be determined by laboratory tests.

6.2. Paraclinical aspect

**** Autopsy and histology***

The presence of the microscopic and gross lesions described earlier will confirm an essentially purulent inflammatory process. Evidence of the presence of *Pasteurella* will be determined by bacteriological testing.

**** Bacteriology***

A serologic diagnosis is rarely made because of the ubiquitous nature of the germ in rabbits. If necessary, an ELISA test can be done (Zaoutis *et al.*,

1991) which is more reliable than precipitation on an agar medium as the hemagglutination inhibition test is not reliable (Kawamoto *et al.*, 1994. The bacteriological diagnosis rather relies on germ culture and identification (Louembe, 1976).

As no specific medium exists for culturing these germs, samples are generally amended on tryptose agar + 5% horse serum. Colonies are then round and iridescent with bluish opalescence and a characteristic smell. When amending is performed on blood agar, there is no iridescent aspect and detection of the presence of *Pasteurella* is much more difficult. In order to identify the germ, it is necessary to examine certain typical biochemical characteristics: MacConkey (-), urea (-), citrate(-), indole (+), glucose (+), sucrose (+). Except for ornitine decarboxylase (ODC) and sorbitol, which can be used as epidemiological markers, the other biochemical characteristics hardly vary in rabbit-isolated *Pasteurella*. ODC determination and evaluation of the colonies' diameter are also pathogenicity markers.

Molecular approach, such as nucleic acid amplification allowed skipping of repeated steps of culture and detecting bacteria directly from clinical samples or early primary cultures, hence reducing the time required for identification. The PM-PCR is based on the amplification of a DNA sequence unique to *P. multocida* (Townsend *et al.*, 1998) and demonstrated a sensitivity of less then 10 organisms.

7. Prognosis

Respiratory forms

In rabbit production, prognosis is cautious in regards to acknowledged cases of pasteurellosis. The prognosis will always be discouraging if *P. multocida* strains are ODC+. It will also be discouraging if environmental conditions cannot be changed sufficiently or at all, and if the producer refuses to cull the diseased animals before implementing treatments. Should a bulk treatment be undertaken, it should be associated with prophylactic measures (Coudert, 1996).

Abscesses

These are economically incurable and for sanitary reasons, animals should be culled as quickly as possible.

For laboratory or pet rabbits, the prognosis will depend on the form under which the disease appears. It will be necessary to implement a heavy antibiotic treatment.

8. Treatments

In vitro trials show that *Pasteurella* are sensitive to a wide range of chemical products. In practice, pasteurellosis curative treatment often proves unsuccessful for the main reason that *P. multocida* is out of reach of the molecules that can be used in animal production (middle ear, abscess, sinus, etc.). The hyaluronic acid present in the germ's capsule might also prevent certain drugs from penetrating into the bacteria (Beckenlehner *et al.*, 1992). Be that as it may, several authors have reported frequent disease relapses following treatments (Broome and Brooks, 1991) or the inefficacy of antibiotic treatments (Jaslow *et al.*, 1981; Mähler *et al.*, 1995).

Should an antibiotherapy be implemented, the parenteral route would be the most appropriate (Hoskins, 1920). Treatment should be applied on a long term basis at high dosage regimens (Laval, 1995). Intramuscular or subcutaneous tetracyclines (oxytetracycline or tetracycline), nitrofuran derivatives, aminoglycosides (streptomycin or DH-streptomycin, gentamicin) and quinolones (flumequine or enrofloxacine) have yielded more or less successful results.

It has been experimentally established that certain antibiotics can have a different efficacy depending on the mode of contamination by *P. multocida* (intradermal or intranasal) (Rideaud *et al.*, 1998). In production breeding, two distinct aspects should be considered: maternity and fattening. In the event of pasteurellosis, treatments administered during the fattening phase will be poorly efficacious in the short term if all control means have not been implemented in the maternity in the first place. In laboratory or pet animals, treatment of systemic affections may be undertaken according to the form present, but failures happen very frequently. In production breeding, only prophylactic measures are economically effective.

9. Prophylaxis

9.1. Sanitary

Similarly to treatments, prophylactic measures will have to be initially implemented in the maternity or doe herd. However, normalising the sanitary situation in the maternity will hardly be of any use if the promoting causes are not reduced during fattening, particularly ventilation and the NH_3 level (Morisse, 1978a, 1978b; Johnson and Wolf, 1993).

**** Offensive methods***

- In the event of endemic pasteurellosis in a rabbitry, depopulation is the most appropriate solution. It is the only solution in the face of abscesses associated with mastitis in does and subcutaneous abscesses in young fattening rabbits (7-8 weeks of age) preceded by diarrhoea episodes.

- In the event of predominantly respiratory chronic pasteurellosis, should depopulation be impossible, it would take several months for the situation to become stable again and, above all, active participation of the producer will be required.

The first precaution consists in choosing 2 or 3 live animals to isolate *P. multocida* strains

(preferably from the middle ear) in order to produce an autovaccine.

The producer should be prompted to immediately prepare a batch of young vaccinated female breeders in other premises. Depending on the severity of the disease, it is necessary to plan a 20-40% renewal of the rabbit colony. This batch of female breeders will enable him to carry out the most important offensive operation more readily: culling diseased animals. Before massively culling diseased animals, the contributing causes must be carefully identified, ventilation must usually be modified and a programme must be set up for cleaning cages, floors and walls.

Poorly productive or unproductive does, which are often a significant source of infectious carriers, as well as older bucks will be culled first. The culling of diseased animals will first be massive (15 to 20% females within 2 to 3 weeks). Note here that the colony restocking rate is approximately 10% per month (Koehl, 1997, 1998). A 20-to-25% colony restocking within a month will be beneficial.

Only after this phase of environmental disinfection will chemoprevention be medically and economically efficacious. If undertaken too soon, it could mask diseased animals that will inevitably relapse and increase the risk of chemoresistance. Administering antibiotics via the diet is rarely efficacious. Injection of long-acting tetracyclines to all breeders at a 2-or-3-day interval will be preferred, while perhaps maintaining breeder vaccination one week after the end of treatments. The literature provides no evidence of the efficacy of vaccinating breeders in the course of production. Restocking with young vaccinated breeders will be preferably carried out after treating maternity breeders in the course of production. The producer will have to be alert for several months, but a simple early culling of the females presenting suppurative coryza will probably be sufficient to stabilise the situation.

**** Defensive methods***

These are appropriate when the situation in a rabbitry is good. In addition to the general rules regarding hygienic prophylaxis, daily activities must be observed. It is essential to cull the animals that are going to be ill. The days preceding parturition are a good period to monitor female breeders (Coudert and Brun, 1989). Examining young rabbits at the time of weaning provides an opportunity to gather information on the dam with regard to her (non)infectious state of harbouring abscess-inducing *P. multocida*.

In the case of colony restocking involving the purchase of selected male breeders, restocking with very young animals will limit the risks of simultaneously introducing pathogenic microflora. Adoption of "day-old" rabbits by dams without clinical pasteurellosis will be preferred whenever the technical level of the rabbitry permits it. In the case of self-restocking, the selection should be drastic and future male breeders withdrawn early from fattening. In addition, apart from conventional hygienic rules, particular attention should be paid to the fact that pelleted feed should not contain too much dust.

Some research laboratories and laboratory-rabbit breeding farmers have *Pasteurella*-free colonies (Scher *et al.*, 1969; Coudert *et al.*, 1988). Extremely strict and radical prophylactic measures are crucial to maintain and keep them in such a state (Weisbroth and Scher, 1969).

9.2. Medical

Vaccination is possible. Numerous experimental works have been carried out, but field results are disappointing (Spanoghe and Okerman, 1987). Although *Pasteurella* immunogenicity is significant, the antibodies produced offer very little protection. The only vaccines yielding less-than-satisfying results are autovaccines. They are used in practice on the sole purpose of restoring a better sanitary situation in the maternity. The best vaccination results are obtained when initial vaccination is performed on 5-to-6-week-old rabbits without simultaneous antibiotic treatment. Two boosters are required at a 4-to-5-week interval. The last booster thus occurs shortly before preparing rabbits for mating. One can see that during the fattening period the acquired protection is notably insufficient.

9.3. Risks for the producer

Pasteurella infections are generally rare in humans. Yet, they can be severe, and even lethal (meningitis). They are usually localised in the oropharyngeal region or are complications of bites, scratches or too-close contacts with pet carnivores, such as cats and dogs (Escande, 1995). In a study carried out in Japan, where pasteurellosis has officially been declared a zoonosis since 1989, Arashima *et al.* (1993) particularly focus on the risk related to close contacts with cats and dogs and show that the frequency of human pasteurellosis has increased in the past few years. A study led in a rural population reports that 32% of traditional breeding farmers are seropositive (Choudat *et al.*, 1987). Furthermore, Le Goff *et al.* (1988) observed that this infection is considered not only as a complication of work injury after bite or scratch, but also as a professional disease entitled to compensation in the framework of the "Régime Agricole" (Agricultural Insurance Scheme) since the recent publication of Table 50 (Official Journal of the French Republic of January 28, 1988).

Donnio *et al.* (1994) reported infectious carriage in the oropharynx of farmers rearing animal species receptive to *P. multocida*: 42% in pig farmers and 10% in cattle farmers versus 0% in rabbit farmers. This last result complies with the fact that no *Pasteurella* infection has ever been reported in

professional rabbit producers. In addition, it supports the fact that although *Pasteurella multocida* can indeed be transmitted from one species to another, each species spontaneously carries different strains.

10. Conclusion

Pasteurellosis is one of the most persistent and constant pathologies in rabbits. It has several forms of manifestation, the most evident of which is the respiratory disease form. However, many other localisations are known and represent equal risk.

Virulence and pathogenicity vary according to *P. multocida* strains. The main reason for pasteurellae persistence in rabbit colonies is probably their localisation in the middle ear. This makes them hard to reach by the molecules used as treatments in animal production. Environmental, particularly ventilation, conditions play an essential role in the outbreak of disease. Therefore, control means should take into account the following two factors: strict culling of carriers (especially of diseased animals) and improvement of environmental conditions. Due to lack of a true and rapidly effective vaccine as a prophylactic measure, hygienic prophylaxis is the most reliable means for controlling respiratory pasteurelloses. Current knowledge does not allow enzootic cutaneous abscesses due to *Pasteurella* to be controlled and, eventually, depopulation is the most economical and effective solution. Finally, although rabbit *Pasteurella* do not seem to be a human health concern, it should be kept in mind that pateurellosis is a zoonosis and that too close contact with cats and dogs is a risk factor for man.

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3.4. Epizootic rabbit enteropathy

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1. Introduction

Digestive diseases are the main cause of morbidity and mortality in growing rabbits (Marlier *et al.*, 2003) and are among those which are the most prejudicial to rabbit farmers. In France, before 1997, the mortality in fattening rabbits was about 11-12% (Koehl, 1997). In 1997 and 1998, it increased to 14-15% and sometimes reached more than 50%, mostly because a new digestive syndrome had emerged. Initially, this new syndrome was wrongly called epizootic rabbit enterocolitis (Lebas *et al.*, 1997). Later, it was more correctly called "epizootic rabbit enteropathy (ERE)" (Licois and Coudert, 2001) because of the absence of any inflammatory lesions on the intestine (Licois *et al.*, 1998).

ERE is characterized by abdominal distension, emission of small quantities of watery droppings which follow a sharp decrease in feed intake, and is highly contagious (Licois *et al.*, 1998). The etiological agent of this emerging disease has still not been identified. However, by using specific pathogenic free (SPF) rabbits and starting from a field sample of intestinal contents of diseased animals, an inoculum (TEC) made up of virulent intestinal contents without opportunistic pathogens such as *E. coli* was obtained (Licois *et al.*, 2005). This reference inoculum has afforded the standardization of experimental reproduction of ERE and a good description of the disease. It has also been shared with another research team that collaborated on etiological research.

2. History

In late 1996 and early 1997, a new severe gastrointestinal syndrome appeared in rabbit farms in the west of France, in Galicia, Spain (Guitian *et al.*, 2000) and probably in Italy (Nieddu *et al.*, 2000). This pathology was characterized by a sudden onset of abdominal distension, emission of small quantities of watery droppings which followed a decrease in feed intake. During this period there were high mortality rates (30 – 80%). The disease spread very rapidly to other regions of France in 1997 and 1998 (Duval, 1998) and to other European countries: Portugal, Hungary, Belgium, The Netherlands, Great Britain, Germany, etc… (Dewrée *et al.*, 2003, Jones and Duff, 2001, Lebas and Coudert, 1997, Rossi *et al.*, 1999). Nevertheless, to our knowledge, this disease has not been reported in any other countries in the world except North Africa (Colin, personal communication).

In France, it is currently estimated that over 95% of farms, are or have been affected by this intestinal syndrome, whatever the rabbit race and strain (Lebas, 2001). Because of the rapid spreading of the disease, it was called Epizootic Rabbit Enteropathy (ERE). The disease mainly affects young fattening rabbits, between six and eight weeks of age. Problems usually occur after weaning but have also been observed in older rabbits, and sometimes in adults or in suckling rabbits, just before weaning. Unlike other epizootic diseases (myxomatosis, viral haemorrhagic disease), wild rabbits do not seem affected even though ERE has been observed in wild rabbit breeding units (Licois *et al.*, 2000).

Up to now, profound changes in the management of breeding, such as generalization of the batch breeding system and improvement of hygienic prevention of diseases, have made it possible to reduce the incidence of mortality.

Nevertheless, ERE is still present in most rabbit farms, but is generally controlled by certain antibiotics.

3. Development of an experimental model

Up to 2001, although experimental reproduction of ERE was successful from a qualitative point of view, since 1997 (Lebas *et al.*, 1997-2001, Licois *et al.*, 2000), we have encountered many difficulties in obtaining constant results from a quantitative point of view. From one trial to another, intensity of the disease (mortality, morbidity, importance of the gross lesions) varied significantly. This variability was also met in the mean time delay in the appearance of the disease after inoculation: 2 to 12 days. To reduce this variability, different methodologies were used and several methods of experimental contamination were tested: immunosuppression of the animals (Licois *et al.*, 1998), intubation via the oesophagus, and pulverization of the inoculum on the animal or on the food. Inoculums of different types were tested (lung, ganglia, mesenteric lymph node, blood), without constant success, with the exception of intestinal contents. Finally, infectious material obtained from various rabbits suspected to be affected by ERE were tested, but were generally found to be contaminated with other pathogenic agents.

Thus, one of the main objectives was to develop a reliable and reproducible experimental model, based on the use of specific pathogen free (SPF) rabbits and on a virulent material originating from intestinal content of contaminated animals. This was achieved in mid 2001, with the constitution of a reference inoculum (TEC1), starting from several samples originating from several protocols carried out with the first sample used as inoculum, known as TEC, kindly provided by P. Hervouet and P. Robart at the end of 1997. These samples came from intestinal contents of about twenty sick or dead animals, between day 3 and day 8 post inoculation (D PI), so that the whole course of the disease was covered (Licois and Coudert, 2001).

Subsequently, other inoculums (TEC2, TEC3) and finally TEC4 (Licois and Coudert, 2005), each derived from its predecessor, were obtained. TEC1, TEC2 and TEC3 inocula were partly characterized at the virological, bacteriological and parasitical level. On direct examination, they were controlled free of coliforms (10^{-1} dilution); the flora was poor and unbalanced, Gram positive bacteria such as *Clostridium* were dominant; however, *Clostridium spiroforme* was not detected. On the other hand, *Clostridium perfringens* belonging to toxinotypes Alpha or Beta2 were identified. Rotavirus tests were negative by ELISA but positive by PCR in all the inocula. PCR tests for other enterotropic viruses (calicivirus, pestivirus, circovirus, adenovirus, coronavirus and parvovirus) were always negative. Inocula were free of intestinal parasites.

In addition, in order to make the samples that could reproduce the disease more representative, two inoculums originating from the Netherlands and from Belgium were prepared in 2002, at the Service of Bird, Lagomorph and Rodent Diseases, Faculty of Veterinary Medicine, University of Liège (Belgium). They were characterized at the virological, bacteriological and parasitic level (Marlier *et al.*, 2003, 2005). They were tested on SPF animals in our experimental facilities and were shown to reproduce ERE (Licois *et al.*, 2003a; Marlier *et al.*, 2005). These samples, originating in the field and obtained outside France, 5 to 6 years after the appearance of ERE in Europe, constitute additional biological material for studies on this pathology, in particular within the framework of the search for the etiological agent.

4. Clinical signs and gross lesions.

In the field, ERE is characterized by sharp decrease in food consumption, one of the first signs observed by breeders, followed by the onset of aqueous diarrhoea and associated high mortality rates (more than 30%) (Coudert *et al.*, 1997; Jobert *et al.*, 2001; Le Gall *et al.*, 1999). Animals are bloated and caecal impaction, associated with presence of mucus in the colon and sometimes in the small intestine, can be observed (Coudert *et al.*, 1997).

However, under field conditions, very few criteria enable a precise diagnosis of ERE, and none of them are specific: increased mortality, presence of mucus under the cages, caecal impaction, are the most common anatomo-clinical signs usually noticed. They are often associated with inefficacy of the usual antibiotics. Moreover, associated or primary pathologies generally hide specific lesions. ERE apparently promoted the development of germs rarely isolated before 1997 (*Klebsiella*, *E. coli* O2, *Clostridium*, etc.) (Barral *et al.*, 2000) and even parasites like coccidia (Coudert *et al.*, 2003a). In addition, the diagnosis is all the more dubious if the general hygienic conditions of the farm are precarious. In the literature, denominations such as “mucoid enteritis”, “mucoid enteropathy”, etc..., are misleading, since mucus excretion as well as caecal impaction in rabbits are physiopathological reactions common to several diseases. In fact, ERE is very close to a disease previously described under the term of mucoid enteritis (Muir, 1943; Hagen, 1956) or mucoid enteropathy (Van Kruiningen and Williams, 1972). The discussion dealing with this nomenclature has been emphasized by Kühn (2005).

Using SPF rabbits and experimental reproduction of the disease, the typical specific clinical signs, macroscopic and microscopic lesions

Figure 1. *Abdominal distension in a five-week-old rabbit that died 4 days after experimental reproduction of ERE with inoculum TEC1.*

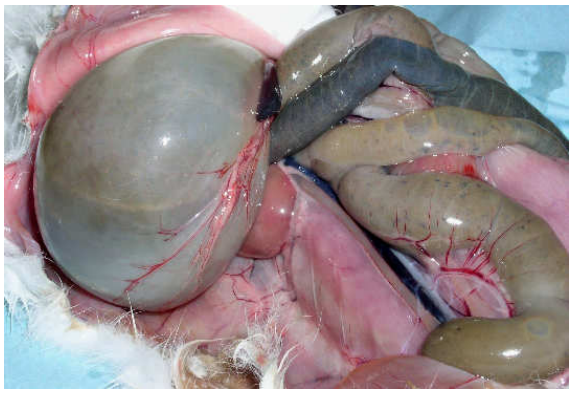

Figure 2. *Six-week-old rabbit that died 5 days after experimental reproduction of ERE with inoculum TEC3. The stomach and small intestine are distended and filled with liquid and gas and are responsible for the bloated abdomen. These lesions, associated with the absence of visible inflammation of the intestinal tract, can be considered as being pathognomonic of the disease.*

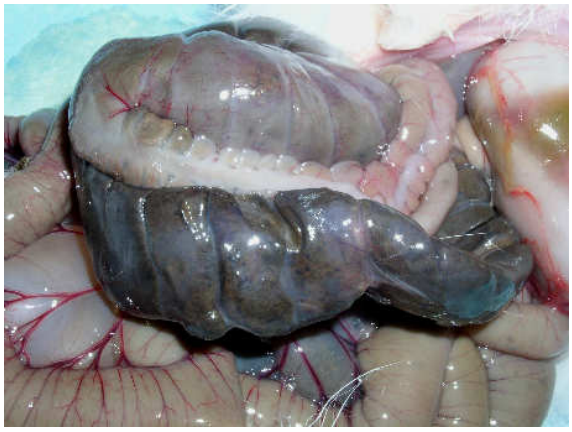

Figure 3. *Total caecal impaction, one of the main gross lesions of the disease in a six-week-old rabbit that died 5 days after experimental reproduction of ERE with inoculum.TEC3. Neither inflammation nor congestion is visible on the caecal wall.*

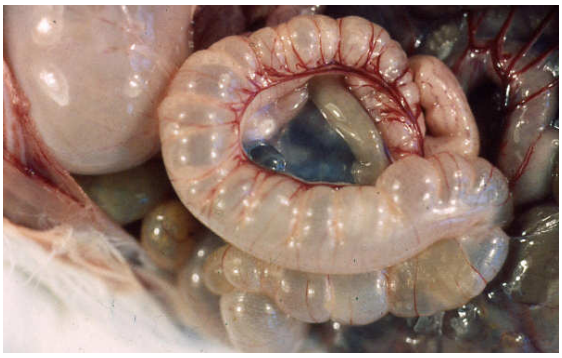

Figure 4. *Proximal colon in a six-week-old rabbit that died 5 days after experimental reproduction of ERE with inoculum TEC3. This intestinal segment is distended by gas and contains mucus (arrows).*

of ERE were characterized, so that the syndrome could be distinguished from clinical signs and lesions of other known intestinal affections. They fully reflect those observed in the field. ERE is characterized by being highly contagious, with a 30 to 40% mortality rate in the first few days, and a morbidity around 100%, whatever the dose of the inoculum used (in a range of 1g up to 50µg of corresponding infectious intestinal contents) (Licois and Coudert, 2005; Licois *et al.*, 2005). ERE can be reproduced in young rabbits aged only 12 days (Boisot *et al.*, 2005) and up to adulthood (Licois *et al.*, 2000). Clinical signs and lesions evolved quickly with the first sign, borborygmus (stomach rumbling) appearing on the first day after inoculation and the disease reaching its crest point 4 to 6 days later. Growth is extremely reduced from the second day to the end of the second week. Rumbling noises and distended abdomen are almost always present, while mucus excretion and caecal impaction are frequent, but not constant. Animals are bloated with watery diarrhoea of low intensity (Figure 1).

Gross lesions are mainly a distension of the whole intestinal tract including the stomach which is filled with gas and fluid (Figure 2). Absence of inflammation or congestion of the intestine is one of the main characteristics. These gross lesions are sometimes associated with caecal paresis (Figure 3) and presence of mucus, especially in the colon (Figure 4). In fact, the digestive changes observed at necropsy obviously indicate physiopathological alterations of the digestive functions. Intestinal transit appears to have been stopped for several days, leading to stomach dilation, small intestine overflowing with large amounts of liquid and gas, and caecal impaction as a final step in paresia of the whole digestive tract. It is worth noting that dilation and accumulation of liquid in the first parts of the digestive tract (stomach, duodenum) have been observed apart from any infectious context, in healthy rabbits following ligature of intestinal loops (Marlier *et al.*, 2003). This highlights the non-specific character of these lesions. Within the EEL

framework, on one hand this could be linked to a putative paralyzing neurotoxin acting at the intestinal level and, on the other hand this paralysis could explain the multiplication of opportunistic germs under field conditions (*Escherichia coli* O2, *Klebsiella*, coccidia,…).

The infectivity of the intestinal contents of inoculated animals as early as the second day post infection was demonstrated (Licois *et al.*, 2005). To better define the precocious events occurring at the onset of the disease, several tests are in progress in our lab. Thus, a severe but transient reduction of growth less than 24 h following administration of TEC inoculum has been observed. Bacitracine antibiotic is unable to correct this initial fall in growth rate, while it does offer good control of the diseases and probably of the pathogen (Coudert and Licois, 2004, 2006). This precocious effect is also observed with the supernatant of the TEC inoculum (Licois, unpublished data, Marlier *et al.*, 2003, Szalo *et al.*, 2006), suggesting the presence of an exogenous toxin in the inoculum which does not lead to pathological consequences when the supernatant alone is used.

From a physiopathological point of view, only slight changes of haematological profiles have been noticed in ERE inoculated animals, with a reduction of blood lymphocytes and an increase of neutrophiles at the peak of the disease (Marlier *et al.*, unpublished data; Jobert *et al.*, (2001); Marlier *et al.*, 2003). On the other hand, it has also been shown elsewhere that the disease does not induce any fever (Jobert *et al.*, 2001).

Finally, regarding immunity, different experiments carried out in our lab have shown that animals that survive the first inoculation with TEC inoculum, become resistant to a challenge with the same product (Licois *et al.*, 2000).

5. Histological study

Since it was really difficult to obtain obvious and reproducible data on histological lesions in field animals, histological examinations of ERE inoculated SPF rabbits was done in collaboration with M. Wyers (Ecole Nationale Vétérinaire de Nantes, France). ERE lesions were compared with those of colibacillosis (EPEC strain O103:H2:K-Rh-) and coccidiosis (*Eimeria media* and *E. magna*) induced also in SPF rabbits.

The two last infectious models (*E. coli* and *Eimeria*) induce almost identical lesions of diffuse, acute to subacute, atrophying, erosive and regenerative enteritis localised in the part of the intestine colonized by these microorganisms (unpublished data). These lesions can be distinguished only through the observation of the specific pathogenic agent, protozoa or Gram- bacilli. In the *E. coli* infection case, intestinal lesions are accompanied by a severe lymphoid depletion of the mesenteric lymph nodes, of the vermiform appendix, spleen and thymus, and are possibly responsible for a transient immunodepression. Coccidia and *E. coli* O103 are also responsible for lesions of similar kinetics, with a peak of lesion severity followed by reduction in intensity and finally persistence of "sequelas", even in the absence of the pathogen involved. These similarities underline, in the absence of the pathogenic agent, the non-specific character of these lesions.

In the case of ERE, the presence of generalized lesions throughout the intestine, with atrophy and fusion of the villi, transepithelial infiltration and migration of viable or pycnotic inflammatory cells were observed in most rabbits (Licois *et al.*, 2005). These lesions are regarded as non-specific. Moreover, a lesional kinetic, characteristic of the development of a pathogenic agent, has never been observed. Lastly, it is important to underline the fact that, although rabbits were carefully selected according to precise symptoms involving ERE, several animals did not present any intestinal lesion. Under these conditions, it is therefore difficult to conclude that a precise kind of pathogenic agent (bacterium, parasite or virus) was involved in the development of ERE. Neither macroscopic nor histological lesions have been observed on the lungs, heart, liver, spleen or kidneys, but an atrophy of the vermiform appendix was sometimes noted.

Therefore, it can be concluded that histology does not offer a satisfactory means of diagnosis. Only some evident criteria of gross lesions, such as the distension of the stomach or of the small intestine, with a liquid content, observed in a sufficient number of animals, associated with parasitological and bacteriological analysis, allows ERE to be diagnosed.

Marlier *et al.* (unpublished data) conducted a study by scanning electron microscopy (SEM) and transmission electron microscopy in SPF inoculated rabbits. SEM performed on the intestinal tract of inoculated rabbits reveals blankets and globular particles of mucus associated with numerous bacteria on jejunum and ileum villi. The presence of bacteria adhering to the epithelial surface and inside epithelial cells in a few animals was demonstrated in the most affected parts of the intestine examined by TEM and by light microscopy after Warthin-Starry staining. Up to now, no link has been established between these bacteria and ERE aetiology.

6. Search for pathogenic agent

6.1. Virus

At the onset of ERE, several arguments supported a viral hypothesis, particularly the epizootic nature and the diffusion of the pathogen, the transmissibility of the disease and the existence of histologically-proven lung lesions suggesting a

viral infection (Wyers, 1998). In addition, most commonly used antibiotic treatments failed to control the disease in affected rabbitries. Thus, since the end of the year 2000, the search for a virus as the ERE etiological agent has been going on. All the common virological analytical methods methods used at INRA (Institut National de la Recherche Agronomique, Tours, France), at AFSSA (Agence Française de la Sécurité Sanitaire des Aliments, Ploufragan, France) and in private firms failed to demonstrate any viral involvement in ERE. The only viruses sometimes found on field samples were rotavirus (Ceré *et al.*, 2000; Marlier *et al.*, 2003) but the disease was never reproduced with the ERE-isolated rotavirus strains. In the same way, the potential role of bacteriophages was considered. Indeed bacteriophages, in particular the caudal bacteriophages, were found in sick rabbits. An experiment conducted at AFSSA failed to reproduce ERE with this type of phages (Le Gall-Reculé *et al.*, 2002 http://www.tours.inra.fr/urbase/internet/resultats/enterocolite/entero1.htm). Szalo *et al.* (2006) conducted some purification steps of the TEC3 inoculum by low speed centrifugation on sucrose discontinuous gradient leading to rotavirus-free fractions which fully reproduced the syndrome, so that they concluded that pure viral aetiology should not be given further consideration.

All the searches conducted for other enterotropic viruses were negative: calicivirus, pestivirus, circovirus, adenovius, coronavirus, parvovirus (G. Le Gall, AFFSSA, 22440 Ploufragan, France). Consequently, at the present time, in the absence of a new element, it has been decided to suspend the research in virology.

6.2. Bacteria

Any aetiological role of enteropathogenic strains of *E. coli* was excluded from the beginning of the studies on ERE. Indeed, despite the recurrent isolation of these bacteria from ERE field cases, no dominant serotypes or EPEC strains were ever found (Marlier *et al.*, 2005). Moreover, most of the field samples we obtained from sick rabbits with ERE clinical signs did not harbour any *E. coli*. The final proof came from ERE experimental reproduction with the TEC inoculum, which are *E. coli* free (Licois *et al.*, 2005). It is worth noting that in direct bacterioscopical examinations the microflora of these inoculums is poor and unbalanced with dominant Gram+, and *Clostridium spiroforme* was never detected (Milon, personnal communication). On the other hand, the implication of other *Clostridium* strains, mainly *C. perfringens* in the genesis of the disease, has been suspected for a long time. The basis for this assertion, is: the similarity of the clinical signs with those of enterotoxemia (dilatation due to gas in the intestine), usual presence of *C. Perfringens* in field rabbits (Dewrée *et al.*, 2003, Le Normand *et a.l*, 2003), *C. Perfringens* strains easily isolated from the inoculum TEC2, TEC3 (Licois *et al.*, 2003a; Marlier *et al.*, 2003), the efficacy of some antibiotics directed against Gram+ bacteria in rabbit farms.

A large-scale bacteriological study was carried out in collaboration with the team of D. Marlier in Belgium, starting from field samples and from inoculum TEC3. A lot of bacterial strains were isolated after aerobic or anaerobic cultures, among them various strains of *Clostridium* including *C. perfringens* (Dewrée *et al.*, 2003; Marlier *et al.*, 2003, 2005). However, no bacterial strains used alone or in a mixture have been able to reproduce the disease (Licois *et al.*, 2003; Marlier *et al.*, 2005). As a result, should ERE be considered a bacterial disease, this putative bacteria would be an uncultivable one or a cultivable one that easily loses its virulence factors (Marlier *et al.*, 2005; Szalo *et al.*, 2006).

C. perfringens strains were isolated in about 80% of rabbits from Belgian or Dutch rabbit farms suffering from ERE (Marlier *et al.*, 2003, 2005). These strains were further characterized (Dewrée *et al.*, 2003; Marlier *et al.*, 2005). Broadly, 66% and 34% of them respectively belong to the A and C toxinotypes. The gene encoding the enterotoxin was identified in 73% of the studied strains. The search by PCR for α, β and β2 toxin revealed that in 86 dead rabbits (32 with symptoms of ERE and 54 with other intestinal pathologies), 69% were positive for α toxin among dead rabbits with ERE, as against 16% among the other dead animals. The gene encoding β2 toxin was only detected in one strain isolated from a dead rabbit with ERE. Le Normand *et al.*, (2003), confirms the prevalence of the α toxin gene among strains (20/38 strains) isolated from dead rabbits with ERE. They indicate also that 17/38 strains were simultaeously αβ2 toxin positive, while none was β positive. These same authors establish a relation between the toxinotype and the clinical aspect of the intestinal lesions. The α toxin positive strains are more frequent in rabbits having liquid caecal content, whereas the strains αβ2 dominate in those having compacted caecal content. This observation has to be confirmed in large-scale field studies. Even if all these studies show the interest in studying *C. perfringens*, the role of this bacteria as primary pathogen will still be questionable. Indeed, no experimental reproduction of ERE has been obtained after inoculation with strains of *C. perfringens* (Licois *et al.*, 2003a, Marlier *et al.*, 2005). Moreover, this bacteria as well as its main toxin were absent from numerous experimental samples originating from the acute phase of successful reproduction of the disease (Marlier *et al.*, 2003, 2005).

However, recent results obtained after fractionation of the inoculum reinforce the hypothesis of a bacterial aetiology of the ERE

syndrome and assign the development of the disease to a particular morphological group of bacteria (Szalo *et al.*, 2006). Finally it has been demonstrated that the unknown pathogen is very resistant in normal environmental conditions (Licois and Coudert, 2005). In experimental conditions the aetiological agent is sensitive to treatment at 80°C, 10 min. either with or without progressive temperature increase, but is resistant to ethanol / chloroform treatment.

6.3. Molecular approach

As we have seen above, works aiming at identifying a virus or a cultivable aerobic or anaerobic bacterium have not yet succeeded. Thus, other avenues had to be explored. A private French company decided to explore a molecular approach based on analysis of nucleic acids originating from sick *vs* healthy rabbits. In order to avoid the use of intestinal DNA and RNA rich in genomes other than that of the organism responsible for the disease, it was decided to carry out sampling of air in the rooms where the animals were placed (although the transmission of ERE by air has never been demonstrated). Samples obtained at various times throughout the experiment were tested for virulence (Licois *et al.*, 2003b) and then chosen for molecular studies, which are still in process (Persillon *et al.*, 2005).

7. Treatment and prevention

Very few antibiotics are efficient in ERE treatment. Tiamulin has a market approval for a 32 ppm use in food, according to French rules. Another molecule, bacitracine has been available since 1998. The effectiveness of Zinc-bacitracin at 100ppm in food has been demonstrated (Duperray *et al.*, 2000, 2003; Approval for Bacivet S® (soluble bacitracin) was obtained in 2005. This last molecule was studied after experimental reproduction of ERE at 0.657g/l of drinking water, before (preventive use) or after (curative use) contamination of animals with TEC3. The results indicate that the preventive use of Bacivet S® is as effective as 100ppm of bacitracine in food used during the acute period of the disease (Boisot *et al.*, 2003a). The curative use of Bacivet S® for 14 days after the onset of symptoms reduces mortality and morbidity compared to control animals, but is less effective than the preventive use. In a field study, Maertens *et al.*, (2005) mention good results obtained with Bacivet S®, better with 21 days of treatment than with 14 days. A field study has also shown that the tylosine (Gram+ spectrum macrolide) could be used as an alternative antibiotic to control ERE (Bostvironnois and Morel Saives, 2003). The best results were found when tylosine was associated with apramycin (Gram- spectrum) confirming previous results on apramycin of Badiola *et al.* (2000). Indeed the development of supervening infections and incidence of complication are very common in field conditions. This is true for bacteria but also for coccidia (Coudert *et al.*, 2000). In a recent epidemiological survey in France it has been shown that a high mortality rate in growing rabbit due to ERE was associated with the highest level of parasitism (Coudert *et al.*, 2003a). In addition, Coudert *et al.* (2003b) have shown that two molecules: salinomycin (against coccidia) and tiamulin (against ERE), can be used simultaneously, without incompatibility, in growing rabbits.

However, for the long term, antibiotherapy is not the best solution and other methods should be used. So nutritional methods are secondary strategies to improve rabbit's health status (Gidenne *et al.*, 2003). Boisot *et al.* (2003b) have shown that in rabbits inoculated with TEC3, a feed restriction of at least 20% below the *ad libitum* consumption level of the control animals, leads to a reduction of mortality and morbidity due to ERE. On the other hand, an attempt to use genetic variability of animals to select rabbits resistant to ERE was carried out by Rochambeau *et al.* (2005) and Garreau *et al.* (2006).

8. Conclusion

Although noticeable progress has been made to better control ERE in the field, the disease is still rampant, with some re-emergence of the problem, particularly in autumn and spring. Efforts must be continued to improve the treatment and prevention of ERE, to improve diagnosis and to identify the etiological agent responsible for ERE. The development of an experimental model and of standardized inoculums has led to better knowledge of the disease, especially during the first hours after experimental reproduction, and to proposing a virulent material which is now used for etiological research in different European laboratories working with this infectious material

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3.5. Rabbit colibacillosis

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1. Introduction

Digestive infections represent one of the main pathological problems in rabbit breeding units, inducing major commercial loss due to weight impairment, epizootic diarrheas, mortalities and veterinary costs. Two main syndromes are responsible for these digestive disorders: the rabbit epizootic enteropathy and the colibacillosis.

Rabbit colibacillosis are consecutive to rabbit infection with highly pathogenic strains, belonging to the enteropathogenic *Escherichia coli* (EPEC) pathovar (Cantey and Blake, 1977). These rabbit EPEC (REPEC) strains have pathogenic mechanisms similar to those described in the human EPEC pathovar. These human strains are responsible for severe epidemic outbreaks of infantile diarrheas in developing countries.

The rabbit strains present a significant epidemiological repartition in western European breeding units. Among this pathovar, strains are classified depending on their O and H antigens expression that defines the serovar of the EPEC strain. In rabbits, several serovars are mainly implicated in diarrhea outbreaks. In France and Spain, the last epidemiological studies reported a predominance of O103:K-:H2 rhamnose-negative *E. coli* (Blanco *et al.*, 1996, Leroy *et al.*, 1994). The O26:H11 isolates also induced severe diarrhea while some other serovars (O128 or O132) induced mild diarrhea and/or weight loss. A recent field study performed in Italy reported that the two main serovars detected are O103 and O2. These two serovars were found in more than 50% of the enteritis outbreaks between 2000 and 2003 (D'Incau *et al.*, 2004). However, no direct correlation between the presence of high rate of O2 serotype and pathogenicity has been shown so far.

2. Clinical signs

The disease mainly affects 4-7 week old rabbits, just after the weaning, and is characterized by severe diarrhea associated with dehydration (Licois *et al.*, 1992). The enteric disease is associated with colonization and proliferation of EPEC in distal ileum, ceacum and proximal colon. With the most virulent strains, animals died within few days. The surviving animals show growth delay. At necropsy, the caecal content appears totally liquid and sometimes haemorrhagic (Fig. 1).

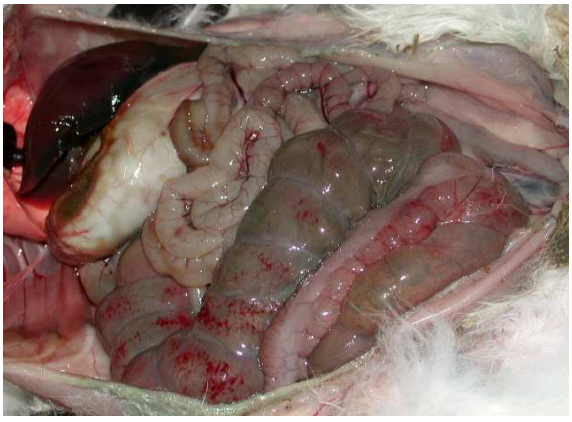

Figure 1. *Intestinal lesions induced by E22 REPEC strain. After E22 induced death, the autopsy reveals an empty small intestine, often distended by gas. The caecum is also distended, with a very liquid content. The caecal wall is thin and haemorrhagic lesions are clearly visible on the epithelium.*

The epithelium is congested. At the tissue level, a strong abrasion of the villi is visible with haemorrhagic area on ceacal epithelium (Peeters *et*

al., 1984b). Experimental infections carried out by Peeters *et al.* have shown that *E. coli* strains involved in rabbit enteritis belong to different serotypes, responsible for different clinical pictures, from weight loss to lethal diarrhea (Peeters *et al.*, 1984a, Peeters *et al.*, 1988).

Interestingly, one EPEC serotype, O109:K-:H2, is mainly associated with yellow diarrhea in suckling rabbits (Peeters *et al.*, 1984a). Animals sensitive to this serotype are very young rabbits (3-12 days old). All the animals of a litter can die within 20 to 48 hours. At necropsy, small intestine and colon are colonized by the bacteria.

Adult animals are usually healthy carriers, and do not present clinical signs after infection. Survival adults, infected after weaning, probably represent the main reservoir of REPEC strains.

3. Virulence mechanisms of EPEC

EPEC strains are characterized by the specific lesion they produce on the host intestinal cell, called the attaching and effacing (A/E) lesion (Frankel *et al.*, 1998). This striking phenotype is characterized by the effacement of microvilli and intimate adherence between the bacteria and the epithelial cell membrane (Moon *et al.*, 1983).

The discovery of distinct plasmid- and chromosome-encoded adhesins in EPEC led to the formation of generalized three-stage model of infection.

The first step of interaction between EPEC and the target cell (either *in vivo* or *in vitro* on cultured cell lines such as Hep-2 or HeLa cells) is a loose of adhesion mediated by specific proteic structures called adhesins. These adhesins usually act as lectins that recognize carbohydrate moieties of glycolipids or glycoproteins on the surface of the eukaryotic cell. The interaction fits the bacteria onto the epithelium. The adhesins are thought to allow colonization of the enteric biotype by the bacteria, which thwarts several non-specific defense mechanisms of the host (such as peristalsis and barrier effects induced by the resident flora). Mechanisms of EPEC adhesion from most of classical serovars have been studied in vitro on cultured epithelial cell lines, such as Hep-2 or HeLa cells. Scaletsky *et al.* (1984) first reported two distinctive patterns of EPEC adherence to HeLa cells : localized adhesion (LA) characterized by bacterial micro colonies attached to discrete zones of the cell surface and diffuse adhesion (DA) where bacteria attached in a scattered pattern to the entire surface. Because of the strong association between LA phenotype and classical EPEC serovars from diarrhea outbreaks, strains giving a LA phenotype are now considered as class I EPEC. Strains showing a DA phenotype or no adhesion to cell lines were less often incriminated in outbreaks of diarrhea and were termed class II EPEC (Levine, 1987, Nataro *et al.*, 1985). The LA phenotype is generated by plasmid borne type IV fimbriae called “bundle forming pili” (BFP), which is exclusively expressed by the E2348/69 human EPEC reference strain and by other typical EPEC of human origin (Giron *et al.*, 1991).

REPEC strains differ from human EPEC strains, in that they do not express BFP or any other identifiable type IV-like fimbriae (Robins-Browne *et al.*, 1994). Instead, REPEC strains produce adhesins that closely resemble K88 fimbriae (Class I adhesins) of another *E. coli* pathovar, the enterotoxigenic *E. coli* (ETEC) (Fiederling *et al.*, 1997, Pillien *et al.*, 1996). The first REPEC adhesin was isolated from the O15 RDEC-1 strain and called AF/R1 for Adhesive Factor/Rabbit 1 (Inman and Cantey, 1983, Inman and Cantey, 1984). However, this adhesin is barely detected in field strains, even in other O15:H- strains (Robins-Browne *et al.*, 1994). A second adhesin was discovered few years later from a highly virulent O103:H2 REPEC strain and called AF/R2 (Milon *et al.*, 1990). Another class I adhesin was discovered in a O15:H- REPEC strain (Adams *et al.*, 1997) and called Ral for REPEC adherence locus. This adhesin is very similar to the AF/R2 adhesin but the localization of its operon is different. Recently, long polar fimbriae were discovered in a O15:H- REPEC strain and were implicated in cell attachment (Newton *et al.*, 2004). These adhesins play an important role in the virulence of the strains but are not sufficient to induce virulence. Indeed, it must be stressed that AF/R2 may also be expressed by some other strains, such as O128 isolates, that are far less virulent than O103 (Milon *et al.*, 1990, Milon *et al.*, 1992). In addition, few highly pathogenic O103 strains express neither AF/R2 nor any other known adhesins (unpublished data). The intervention of other specific adhesins cannot be ruled out and has to be searched for, including in the O103 group.

Once the bacteria are loosely attached to the membrane of the host cell, several genes of virulence are activated and bacteria closely attached to the host cell. This is the second step of the infection.

This intimate attachment is mediated by another adhesin, intimin (Jerse *et al.*, 1991) and is associated with the typical “attaching and effacing” lesion, characterized by the loss of the enterocyte microvilli and intimate adherence (Knutton *et al.*, 1989, Finlay *et al.*, 1992). Intimin mediates close contact between the bacteria and the target cell upon interaction with its translocated receptor Tir (for Translocated intimin receptor) (Kenny *et al.*, 1997). All the genes necessary for the A/E lesion formation are encoded within a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). The LEE and the A/E ability are also present in

enterohaemorrhagic *E. coli* (EHEC) strains, some strains of *Hafnia alvei* and *Citrobacter freundii* biotype 4280 (Frankel *et al.*, 1994, Schauer and Falkow, 1993). The complete sequence of the LEE from the human EPEC reference strain E2348/69 has recently been published (Elliott *et al.*, 1998). Several lines of evidence indicate that the LEE has been horizontally transferred on several occasions within *E. coli* clones, therefore giving rise to several lineages of EPEC and EHEC. They differ by the insertion site, length of the locus and/or alleles of some genes (Perna *et al.*, 1998, Sperandio *et al.*, 1998, Wieler *et al.*, 1997).

Several laboratories have studied the interaction between intimin and its receptor. The C-terminal 280-amino acid domain of intimin supports the interaction site with the receptor (Frankel *et al.*, 1995) and shows high polymorphism that leads to the recognition of at least ten subgroups within EPEC and EHEC, irrespective of the strain origins and pathotypes (Adu-Bobie *et al.*, 1998, Agin and Wolf, 1997, Torres *et al.*, 2005). In addition, several studies suggest that Tir is perhaps not the only receptor of intimin and may act as a co-receptor with the help of eukaryotic molecules such as β1 integrins (Frankel *et al.*, 1996a) or nucleolin (Sinclair and O'Brien, 2004). The importance of the different intimin types in colonization of the intestine has been emphasized, with recent data suggesting that differences in the amino acid sequences of the intimin proteins influence the pattern of colonization and tissue tropism in the host (Phillips and Frankel, 2000).

Besides Tir and intimin, the LEE encoded proteins with a wide range of functions, including a type III secretion system (TTSS), various secreted effector proteins and their chaperones (Donnenberg *et al.*, 1997, Elliott *et al.*, 1998).

Tir is secreted by the type III secretion system (sec/esc machinery) and translocated into the host cell membrane of a bacterial syringe constituted by at least three different molecular effectors, EspA, EspB and EspD (Kenny *et al.*, 1997, Elliott *et al.*, 1998) (Figure 2). EspA is thought to be a structural protein and forms the body of the syringe (Ebel *et al.*, 1998, Knutton *et al.*, 1998). EspB would be at the end of the syringe, forming a pore in the cell membrane (Hartland *et al.*, 2000) but would also be translocated into the cell cytoplasm and the cell membrane (Knutton *et al.*, 1998, Taylor *et al.*, 1998, Wolff *et al.*, 1998) where it interferes with normal cell metabolism (Taylor *et al.*, 1999, Savkovic *et al.*, 1997). EspA, EspB and EspD are all required for signal transduction and the formation of the A/E lesion.

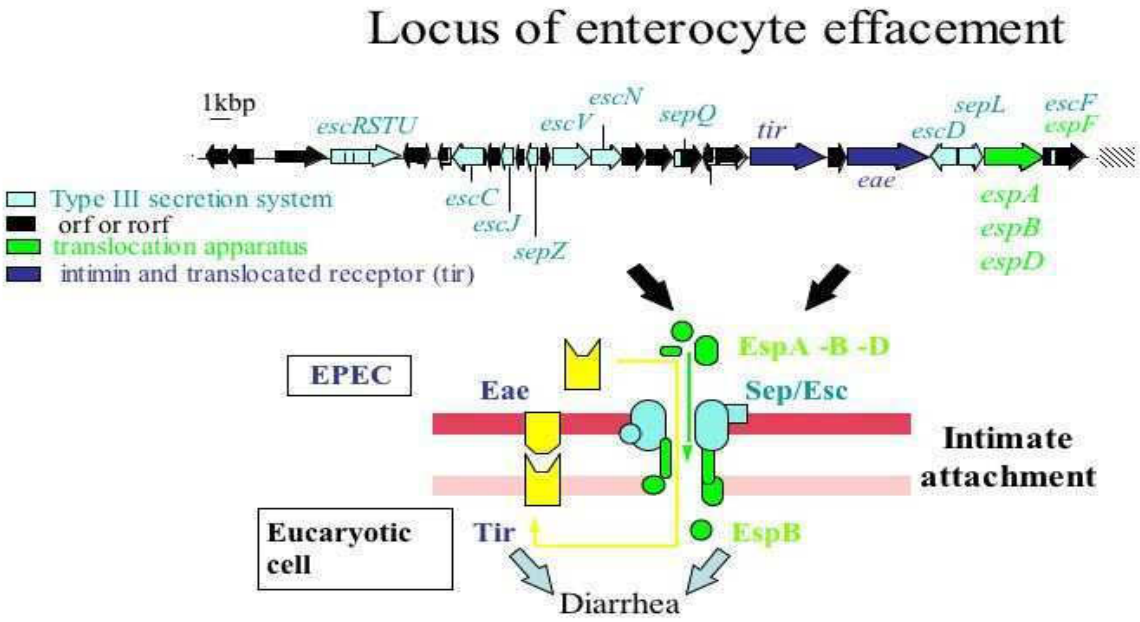

Figure 2. *Schematic representation of the LEE. The type III secretion system allows the secretion of the translocation apparatus that forms a molecular syringe into the host cell membrane. Several bacterial effectors are directly injected into the cell host cytoplasm through this syringe. Tir is expressed on the host cell surface and interacts with the bacterial adhesin intimin.*

After its injection into the cell cytoplasm, Tir is tyrosine-phosphorylated inside the target cell and bound to the cell membrane. Then, the complex intimin/Tir triggers or subverts signal transduction pathways and acts as an organizer for cytoskeleton proteins nucleation and pseudopod formation

beyond intimin receptor (Kenny and Finlay, 1997, Rosenshine *et al.*, 1992; Rosenshine *et al.*, 1996). It leads to the disappearance of microvilli and the formation of the pedestal structure beyond the site of bacterial attachment, characteristic of the A/E lesion (Licois *et al.*, 1991, Peeters *et al.*, 1984a). Tyrosine phosphorylation also activates host phospholipase C (Foubister *et al.*, 1994), which leads to the elevation of intracellular free calcium level (Baldwin *et al.*, 1991) and to the activation of serine or threonine kinases and subsequent phosphorylation of different host proteins (Baldwin *et al.*, 1990, Manjarrez-Hernandez *et al.*, 1992). Phosphorylation of membrane proteins may be responsible for ion secretion and subsequent diarrhea (Law, 1994). Other virulence factors are injected into the cell cytoplasm by the type III secretion system and the injectisome. Among them, EspF (also encoded within the LEE) disrupts tight junctions and favors epithelial cell desquamation (McNamara *et al.*, 2001) and Cif blocks the cell cycle (Marches *et al.*, 2003). All these perturbations of cell metabolism represent the third step of EPEC virulence. Diarrhea probably results from multiple mechanisms, including active ion secretion, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvilli effacement.

4. Immune response

Little is known about the host immune responses to EPEC. EPEC have been shown to have pro-inflammatory activities through the activation of IL-8 synthesis by epithelial cells (Savkovic *et al.*, 1997). Among the different possible bacterial effectors responsible for this inflammatory response, different studies indicate that intimin induces the synthesis of pro-inflammatory cytokines (Ramirez *et al.*, 2005) and orients the immune response toward a Th1-1 profile (Higgins *et al.*, 1999).

In parallel, it has been shown that EPEC strains could have immunomodulatory properties. It has been shown that EPEC lysates could interfere with the synthesis of interleukine-2 (IL-2), IL-4, IL-5 and gamma -interferon production by mitogen – activated human blood mononuclear (Klapproth *et al.*, 1995). The same results were found for human gastro-intestinal lymphocytes (Klapproth *et al.*, 1996) and murine splenic or mucosal lymphocytes (Malstrom and James, 1998), suggesting that protein(s) from EPEC could suppress specific local immune responses. One virulence protein candidate is lifA, a toxin encoded outside the LEE that inhibits the transcription of multiple cytokines and inhibits lymphocyte proliferation (Klapproth *et al.*, 2000).

The protective immune response is probably associated with the presence of specific antibodies (Abs) at the intestinal surface. In a vaccine assay, we demonstrate the presence of anti-AF/R2 IgA Abs in the sera of rabbits vaccinated with a live attenuated AF/R2 positive strain (Boullier *et al.*, 2003a). These Abs were able to inhibit bacterial attachment to epithelial cell in vitro (Boullier *et al.*, 2003b). Similarly, it has been shown that local anti-AF/R1 IgA interfere with the adhesion of RDEC-1 on the intestinal epithelium and facilitate the resolution of infection (McQueen *et al.*, 1992). Antibodies against other virulence factors could also be important for the protection. Several studies indicated that after EPEC infections, antibodies against Esp proteins and Tir are detectable in the colostrum of patients (Camara *et al.*, 1994; Camara *et al.*, 1995, Loureiro *et al.*, 1998, Sanches *et al.*, 2000). The presence of these Abs could explain the relative resistance to EPEC infections of breast-feeding children. Other studies emphasize the role of the anti-intimin Abs in the protection of infected patients (Manjarrez-Hernandez *et al.*, 2000, Frankel *et al.*, 1996b, Ghaem-Maghami *et al.*, 2001). However, the duration of the protection and the cross protection between different serovars are still unknown.

5. Treatment and Prevention

The use of antibiotics to treat or prevent EPEC colibacillosis is not easy, owing to the peculiarities of the digestive physiology and of the normal flora of the rabbit on one hand, and to the frequent acquired multiple resistances of *E. coli* strains, on the other hand. In addition, the massive use of antibiotics stressed the problem of food residues. EU policy tries to put a ban on such practices, and consumers as well as breeders ask searchers to elaborate alternative breeding strategies that improve the health status of animals, without using in-feed antibiotics. Furthermore, it must be kept in mind that wide spectrum antibiotics, such as ß-lactames (ampicillin, amoxicillin, cephalosporins ect…), lincomycin, clindamycin are highly toxic for the rabbit, essentially because they induce tremendous disequilibrium of the digestive flora (usually associated with death). Narrow spectrum antibiotics, such as polypeptides (flumequine, enrofloxacin) may help to decrease mortality during epizooties. However both field and experimental data show that antibiotics used in supplemented feed or by individual administrations cannot really solve the problems linked with EPEC infections in rabbits. In addition, EPEC strains show high frequency conjugative plasmid borne resistances that may be selected by *in vivo* use of relevant antibiotics and which may spread easily in the enteric population (Blanco *et al.*, 1994, Reynaud *et al.*, 1992).

Other means of specific and non-specific therapy and prevention have been tested in the rabbit EPEC colibacillosis.

In our laboratory, we tried to prevent weaned rabbit colibacillosis by vaccination. Owing to the epidemiological predominance of O103 strains in France, we focused the study on this type of REPEC. Different vaccination studies done in our laboratory have given encouraging results. During the first vaccination trials, rabbits inoculated with high doses of formaldehyde-killed whole bacteria were protected against a virulent challenge (Camguilhem and Milon, 1990). However, the heaviness of the protocol and the high doses to be used led to the search for another vaccine strategy. We demonstrate that protection against EPEC O103 infections could also be induced by oral administration of live *E. coli*. The weakly pathogenic strain C6 (O128, LEE and AF/R2 positive) protects at least partially weaned rabbits from REPEC O103 and induces local IgA anti-LPS responses (Milon *et al.*, 1992). With the discovery of the LEE and the better understanding of EPEC virulence mechanisms, we constructed a live attenuated strain from the virulent O103:K-:H2 REPEC strain. We inactivated two virulence genes *espB* and *tir*. Our vaccine is totally safe and induces a specific immune response. The vaccine protects rabbit from a homologous virulent challenge after a single oral inoculation dose (Boullier *et al.*, 2003a) (Figure 3). Recently a new REPEC vaccine has been constructed. The vaccine was obtained by deleting the LEE encoded regulator (ler) of the EPEC strain, which reduces the synthesis of LEE encoded proteins. This strain was totally safe and protects rabbits from a homologous virulent challenge (Zhu *et al.*, 2005). In both studies, it remains to be determined whether such mutants can protect from infection with A/E bacteria of differing serotypes.

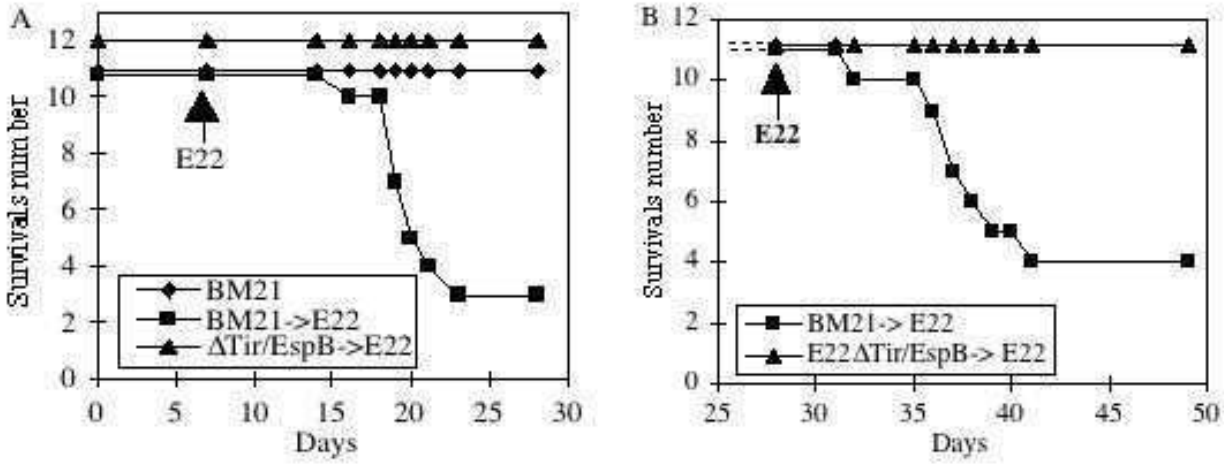

Figure 3. *E22Δtir/EspB strain protects rabbits against an E22 virulent challenge*

A: E22Δtir/EspB vaccinated and non-vaccinated rabbits were inoculated with E22 7 days after vaccination. Three among 11 non-vaccinated rabbits died within 10 days post inoculation while all vaccinated rabbits survived and did not show any clinical sign.

B: E22Δtir/EspB vaccinated and non-vaccinated rabbits were inoculated with E22 28 days after vaccination. Four among 11 non-vaccinated rabbits died within 12 days post inoculation while all vaccinated rabbits survived and did not show any clinical sign.

Another strategy to improve the resistance of just weaned rabbits to colibacillosis but also to other infectious intestinal diseases would be to improve their non-specific immune resistance by favoring the installation of microflora barriers. Indeed, a new concept of potentially beneficial intestinal microorganisms called probiotics emerged a few years ago. The definition of probiotics states: "oral probiotics are living microorganisms which, upon ingestion in specific numbers, exert health benefits" (Guarner and Schaafsma, 1998). Probiotics consumption is reported to exert their beneficial effects through several ways including balancing of colonic microbiota and enhancing immune responses (Heyman, 2000, Kaur *et al.*, 2002). Probiotics are mainly bacteria and yeast naturally present or not in the resident microflora. At the present time, in European union, only *Bacillus cereus* (var toyoi or not) and *Saccharomyces cerevisiae* are authorized for reproductive does or growing rabbits. In this species, probiotics are used to improve zootechnical performances of females or growing rabbits. No direct studies on the role of probiotics on the immune system have been performed so far in the rabbit species. However, several microorganisms have been shown to have potential probiotic activities against EPEC strains in rabbits. In infants'

rabbits, it has been shown that, after infection with an O157:H7 strain, daily oral administration of *Lactobacillus casei* strain decreased the severity of diarrhea and the level of intestinal colonization by the pathogenic strain (Ogawa *et al.*, 2001). Another study shows that colonization of the rabbit intestine by segmented filamentous bacteria inhibits the attachment of an O103 REPEC strain to the epithelium and protects from colibacillosis (Heczko *et al.*, 2000). It has been shown that addition of *Saccharomyces boulardii* to epithelial cells in vitro prevents EPEC induced cell signal transduction (Czerucka *et al.*, 2000). It would thus be interesting to search for specific rabbit probiotics in order to increase their resistance to EPEC infections.

A third strategy to improve rabbit resistance to colibacillosis would be to modify breeding techniques, especially around weaning. Indeed, the weaning period represents the main sensitive period to colibacillosis of rabbits. In collaboration with the INRA SRC group (directed by T. Gidenne), we showed that young rabbits are protected against EPEC infection as long as they receive maternal milk. The protection stops with the weaning (personal communication). The main hypothesis set up by this result is the presence of one or several anti-microbial factors in the doe milk, different from specific antibodies. Several studies have already demonstrated the presence of anti-microbial components in the milk of other mammal species, able to inhibit EPEC virulence in vitro. Ochoa *et al.* (2003) shows that lactoferrin impairs type III secretory function in EPEC strains *in vitro*. Rhoades *et al.* (2005) reports that caseinoglycomacropeptide (derived from kappa-casein) inhibits cell adhesion of EPEC strains *in vitro* (Rhoades *et al.*, 2005). Several studies reported that fat components of the milk also display anti-microbial properties (Isaacs *et al.*, 1995). More precisely, short chain fatty acid (C8 and C10) have been shown to have *in vitro* anti gram-negative properties (Sun *et al.*, 2002) and interestingly, doe milk is extremely rich in C8 and C10 short chain fatty acid. It would thus be interesting to further explore the anti-microbial properties of milk doe. It would thus be possible to test the effect of complementation of food with this anti-microbial component(s) on the resistance to colibacillosis of new-weaned rabbits.

6. Conclusion

Colibacillosis remains a major economical problem in rabbit breeding units. The use of antibiotics (in prevention or in treatment) does not represent an efficient and adequate solution. Numerous laboratories have studied EPEC virulence factors and the pathogenicity of the bacteria is now well known. These discoveries have allowed the development of live attenuated vaccines. However, little is known of the specific immune response against EPEC, with regard to the nature of the protective antibodies and to the duration of the immunity. The obtaining of these data should improve the quality of the vaccines.

In parallel, other strategies, like the use of probiotics and new breeding systems, to prevent colibacillosis and other digestive infections should be considered.

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3.6. Viral enteritis of rabbits

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1. Introduction

In the group of diseases characterising the present situation of the rabbit industry, the so-called "multifactorial conditioned diseases" are really very important.

The "multifactorial enteropathy" is the most important of these technopaties, especially in relation to its productive and economic impacts. It is a pathologic complex of the growing rabbit, known also as "Enteric syndrome", characterized by a great number of stressors and pathogens acting in synergy with a different degree of virulence i.e. various aetiological agents (viruses, bacteria and protozoa) can act together to cause tissutal damage at the gut level, thus determining severe diarrhoea and malabsorption.

The seriousness of this bowel pathology is related to the losses it is able to cause, either direct due to the death of growing rabbits, or indirectly because of a reduced or absent growth and above all the high rate of discarded animals.

The unpredictable appearance and the clinical variability, as well as the variable pattern of pathologic lesions, make epidemiological, diagnostic and laboratory research very difficult. Post mortem lesions are not typical and many pathogenic agents are often involved at the same time, or they follow each other, and consequently their real pathogenic role is still uncertain. On the contrary, it is well known that many conditioning factors (wrong feed formulation, decreased food taking because of adverse seasonal conditions, managerial mistakes, excessive antibiotic administration, loss of passive immunity, cold, early weaning) are involved in inducing this syndrome by promoting the overgrowing of primary or potential pathogenic agents and/or increasing rabbit sensitivity (Lelkes et Ghang, 1987).

It is commonly accepted that rabbit enteric viruses can act as potential pathogens and therefore the presence of viruses in association with particular conditions can induce enteric problems.

2. Rotavirus

Among the viral agents identified in rabbits with enteric disease, Lapine Rotavirus (LRV) is certainly the more widespread agent even if it is considered only mildly pathogenic (Thouless *et al.*, 1988). It primarily causes enteric disease and is detected in post weaned rabbits but it could also be involved in the aetiology of more severe enteriris outbreaks in association with *E. coli*, clostridia and protozoa.

Rotavirosis in rabbits is usually caused by a Group "A" rotavirus (Figure 1). Several strains with different antigenic and genomic properties have been described (Martella *et al.*, 2004; Martella *et al.*, 2005). The LRV infection is characterized by a high rate of morbidity and clinical signs (i.e. diarrhea, anorexia, depression vomiting) and it mainly occurs in fattening rabbits, 4-8 weeks old but early infection in lactating rabbit of 8-21 days old as well as late reinfections in 10-12 weeks old rabbits are possible.

Rabbits can become infected by the oro-fecal route and the extension and the severity of the lesions are dose dependent i.e. the consequences of the infection (micro-villus degeneration, malabsorption and diarrhoea) are higher when the infectious dose is also high. Rotavirus was detected in 16.4% of post-weaned rabbits with enteric signs (Nieddu *et al.*, 2000) and, according to our data

Table 1. Distribution of viral positivity for year and type of virus, detected by EM during the period 1997-2005 from rabbits with enteritis

Year	Total	Negative		Rotavirus		Parvovirus		Coronavirus		Enterovirus		Adenovirus		Calicivirus		Reovirus	
	cases	n.	%	n.	%	n.	%	n.	%	n	%	n.	%	n.	%	n.	%
1997	82	52	63.4	19	23.2	6	7.3	10	12.2	2	2.4	2	2.4	0	0.0	0	0.0
1998	98	63	64.3	20	20.4	8	8.2	10	10.2	3	3.1	0	0.0	0	0.0	0	0.0
1999	59	37	62.7	12	20.3	2	3.4	8	13.6	0	0.0	0	0.0	0	0.0	0	0.0
2000	49	38	77.5	5	10.2	6	12.2	6	12.2	1	1.2	0	0.0	0	0.0	0	0.0
2001	73	48	65.7	9	12.3	20	27.4	12	13.7	1	1.4	1	1.4	0	0.0	0	0.0
2002	35	17	48.6	9	25.7	5	14.3	8	22.9	2	5.7	1	2.9	1	2.9	0	0.0
2003	34	18	52.9	8	23.5	4	11.8	8	23.5	3	8.8	0	0.0	1	2.9	0	0.0
2004	46	27	58.7	4	8.7	3	6.5	12	26.1	5	10.9	0	0.0	0	0.0	0	0.0
2005*	39	18	46.2	9	23.1	5	12.8	10	25.6	1	2.6	0	0.0	0	0.0	1	2.6
Total	515	318	60.0	95	18.6	59	11.5	84	18.1	18	4.1	4	0.7	2	0.6	1	0.3

* updated at 30 september 2005

such incidence has almost been the same (range 8.7-25.7) for many years (Table 1). However, seroepidemiological surveys have shown that most adult rabbits are seropositive for rotavirus, thus indicating that there is normally a constant circulation of low amounts of rotavirus in industrial rabbit farming (Peeters *et al.*, 1982: Peeters *et al.*, 1984; Di Giacomo *et* Thouless, 1986).

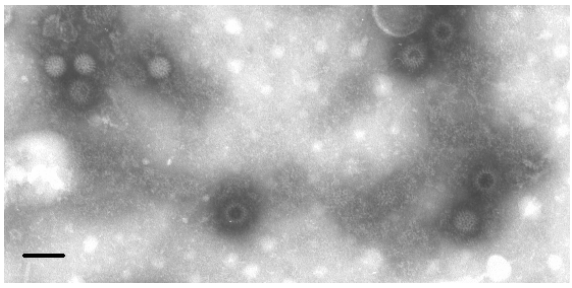

Figure 1. *Negative staining electron micrograph of rabbit rotavirus. Full and empty particles are visible. Bar=100nm*

Healthy rabbits became subclinically infected mostly at 4 weeks of age with viral particles excretion for only 3 days. Rabbit with clinical signs continue to eliminate virus for 6-8 days. The persistence of maternal antibodies until 30-45 days can reduce the symptoms of the disease. Diarrhoeic symptoms appear at the beginning of viral excretion and they are generally followed by stipsi. Lesions observed at necropsy are not constant: catharral, haemorragic or necrotic enterotyphlitis and caecal impaction.

Virological diagnosis can be achieved by testing faeces and intestinal contents by ELISA, electron microscopy (E.M.) and PCR.

The introduction of breeders of unknown origin, without the application of a quarantine period is an important risk factor. Thus, a reduction in biosecurity and hygienic activities (cleaning, disinfection, removal of litter, within others) can lead to a huge increase of the environmental contamination with rotavirus.

Even if rabbit rotavirus is considered only mildly pathogenic, meat rabbits suffering from enteritis can die due to dehydration and secondary bacterial infection. In those that recover, a decrease in productivity is commonly observed due to reduced absorption capacity (Thouless *et al.*, 1988; Thouless *et al.*, 1996; Schoeb *et al.*, 1986).

3. Coronavirus

Rabbit Coronavirus (RbCoV) is not yet definitively classified, and it is an unassigned member in the Coronavirus genus. Coronavirus particles are described as agent of two different pathologic forms in rabbit: a systemic disease (known as pleural effusion disease or cardiomyopaty of rabbit) and an enteric disease (Lapierre *et al.*, 1980; Osterhaus *et al.*,, 1982). The systemic disease is characterized by fever, anorexia, leucocytosis, lymphocytopenia, anaemia, hypergamma-globulinemia, iridocyclitis, which are often followed by death. The lesions are often localized to myocardial and pleuric level. The enteric disease shows the lesions and symptoms typical of enteritis caused by coronavirus in other species. The virus first observed at E.M. in faeces of rabbit with diarrhoea and its antigenic and structural properties were then determined (Descoteaux *et al.*, 1985). It replicates in small intestine with necrosis of apical villi followed by diarrhoea (Descoteaux *et* Lussier, 1990).

A high prevalence has been found in seroepidemiological surveys (Deeb *et al.*, 1993), indicating a wide diffusion of the RbCoV in rabbitries. Diagnosis of coronavirus could be done by using negative staining electron microscopy (Figure 2).

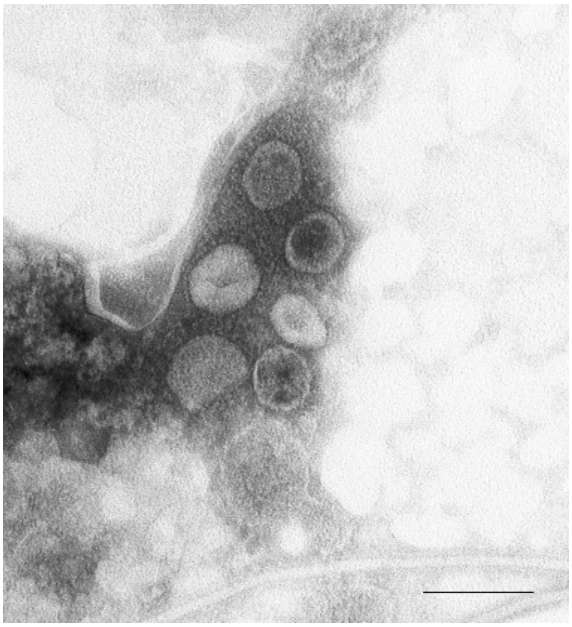

Figure 2. *Negative staining electron micrograph of rabbit coronavirus particles. Bar=200nm*

Just the important enhance of coronavirus-like positivity (Table 1) suggested improving the study on this agent, whose role as enteric and/or systemic pathogen is not yet completely cleared. A first serological surveys performed in three rabbitries, using an indirect ELISA based on the used of cross-reactive reagents for bovine coronavirus (BCV), indicated a widespread seroprevalence. However, by testing with a sandwich ELISA for BCV 16 samples resulted positive at EM for coronavirus-like particles, we only detected a faint positivity in 6 of them. We also tried to isolate *in vitro* the virus and to define its haemoagglutination properties: rabbit coronavirus, similar to bovine BCV seems to grow

in HRT18 cell line and it haemogglutinates mouse red blood cells but not those of rabbit.

In our surveys coronavirus was frequently associated with other viruses, and mainly with rotavirus, accounting 80% of association during the period 2002-2004 (Table 2 and Figure 3), so it could be possible that it can bring together viral and bacterial agents to determine post-weaning enteritis. Therefore, the pathogenic role as the cause of primary enteric disease was not evident and clear but the spread of virus and its high seroprevalence (100% farms, 3-40% rabbits) suggest the possibility of subclinical infection and a probable role as opportunistic pathogen.

Table 2. Type and number of viral associations detected by EM during the period 2002-2005 from rabbits with enteritis

Type of association	2002	2003	2004	2005*	Total
Parvovirus + Enterovirus	1	0	0	0	1
Rotavirus + Enterovirus + Coronavirus	0	0	1	0	1
Rotavirus + Coronavirus	3	3	0	3	9
Enterovirus + Coronavirus	0	1	1	0	2
Parvovirus + Coronavirus	1	2	1	0	4
Rotavirus + Calicivirus	0	1	0	0	1
Rotavirus + Enterovirus	1	0	0	1	2
Rotavirus + Coronavirus + Calicivirus	1	0	0	0	1
Reovirus + Coronavirus	0	0	0	1	1
Rotavirus + Parvovirus	0	0	1	0	1
Total	7	7	4	5	23

* updated at 30 september 2005

4. Other viruses

The rabbit parvovirus (Figure 4), first described by Matsunaga *et al.* (1977), has a very low pathogenicity and it is commonly isolated from the gut contents of healthy animals. It could cause very mild clinical signs (lethargy, disorexia and depression) in experimentally infected animals and a mild to moderate enteritis in the small intestine (Matsunaga et Chino 1981). Its primary pathogenic role is still unclear but considering its frequency of identification (Table 1), it could be important just in multiple infections together with other infectious agents (viruses, bacteria and parasites).

Some of the other viruses detected during diagnostic activity (Table 1) had only a sporadic occurrence so their pathogenic role is probably negligible. Adenovirus has been previously reported only once (Bodon et Prohaszka 1980). Reovirus and enterovirus have never been described before as enteric agents of rabbits. However we can not exclude that enterovirus-like particles correspond to the picobirnavirus (Gallimore *et al.*, 1993), stating the strict morphological similarities existing with this group of non cultivable RNA viruses, identified in several species (humans, pigs, chickens, guinea

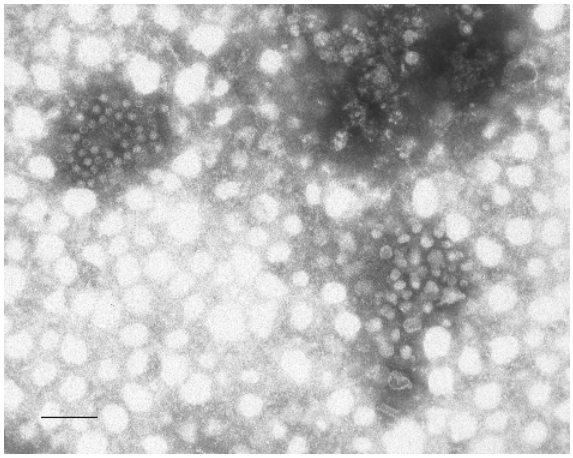

Figure 3. *Negative staining electron micrograph of rabbit parvovirus (group on the left) associated to coronavirus particles (group on the right). Bar = 200 nm*

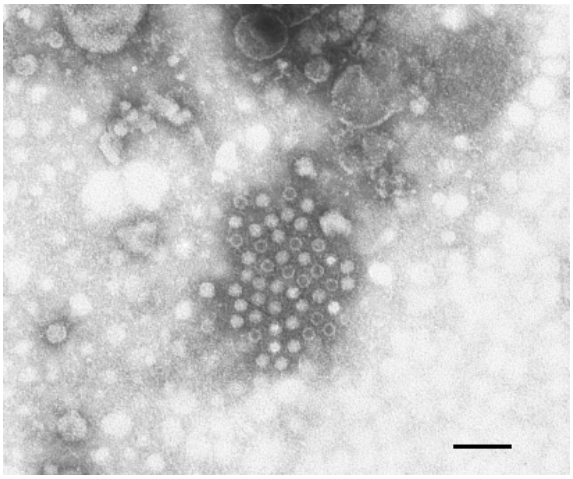

Figure 4. *Negative staining electron micrograph of rabbit parvovirus. Viral particles are clumped by using a specific hyperimmune. Bar=100nm*

pigs) including rabbits. Lusert *et al.* (1995) found that picobirnavirus were commonly excreted by 10% of rabbits without causing any symptom or lesions. A cultivable calicivirus, genus vesivirus, has been recently identified in juvenile growing rabbit showing symptoms of diarrhoea (Martin-Alonso *et al.*, 2005) and it was shown to be neither related to Rabbit Haemorrhagic Diseases virus (RHDV) nor to Rabbit Calicivirus (RCV).

5. Conclusions

Among the different pathogens that could be found in rabbits suffering from enteropathy, viruses seem to have and important but not definitive role. Viruses, and rotavirus particularly, are not able to induce primary episodes of high gravity but, acting as mild pathogens, they could have the capacity of becoming endemic.

The situation of intensive rabbit-breeding, is characterised by intense genetic selection, exasperated productive performances, sometimes overpopulation and consequently high environmental pollution of facultative pathogens. Therefore, these viruses and other low pathogenic agents (es. flagellata protozoa), can explicate a more important role for the occurrence of severe enteritis in rabbit, predisposing and aggravating secondary microbial infections.

One possibility, already proposed by others, is that they can primarily cause damage on the intestinal mucosa thus predisposing the attachment and replication of bacteria. In such case it does not exclude a dose dependant effect, nor a transient infection and a short period of excretion, thus making possible the detection of viruses in association with the presence of *E. coli*, *Clostridium* spp, Coccidia and other protozoa.

On the other hand we can't exclude that the changed physiological and metabolic conditions, induced to enteric level by various factors both alimentary or not, can enhance the replication of viruses normally present at a lower concentration, permitting them to explicate a pathogenic action.

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3.7. Rabbit haemorrhagic disease (RHD)

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1. Introduction

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal acute hepatitis, of wild and domestic European rabbits (*Oryctolagus cuniculus*).

RHD was first reported in 1984 in the People's Republic of China [Liu *et al.*, 1984] and in Europe two years later enormous damages was caused to the rabbit industry, at least till the development of an inactivated vaccine and introduction of its use in prophylactic programs. RHD has a high rate of diffusion; in fact, outbreaks of RHD have been reported in over 40 countries in North and South America, Africa, Asia and Europe. As a rule, the presence of RHD as an endemic disease in several areas is the consequence of the presence of steady European rabbit population (i.e the contemporary presence of wild rabbits, familiar rabbitries and industrial farms). Of course, in spite of the availability of an effective vaccine, the goal of the eradication of RHD in these areas is very difficult to accomplish. Finally, RHD has been intentionally introduced in Australia and New Zealand (Cooke et Saunders, 2002), where rabbits cause serious ecological and economic problems and are considered a pest, in order to keep the level of rabbits reproduction as low as possible. From a pure scientific point of view, it will be very interesting to follow the evolution of the relationship between rabbits and the virus that cause RHD (RHDV) in Oceania, in comparison with the previous experience of the deliberate introduction of the Myxomavirus. One of the main questions is: will RHDV, a small round RNA virus, evolve in less virulent strains and in resistant populations of rabbits as occurred with the Myxomatosis virus, a large DNA virus?

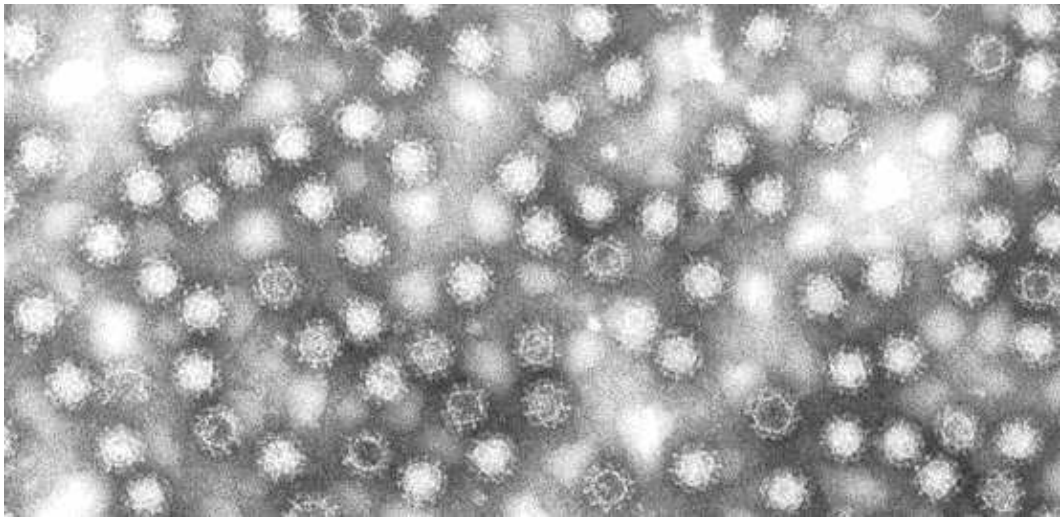

Figure 1. *Electron micrograph of RHD purified virions*

2. Characteristics of the causative agent

The causative agent of RHD (Figure 1) is a Calicivirus classified, together with the European brow hare syndrome virus (EBHSV), in the genus Lagovirus. It is 32–35 nm in diameter and has a single major capsid polypeptide (60 kDa), a positively stranded RNA genome of 7437 kb and a sub-genomic RNA of 2.2 [Meyers et al., 1991a, b; Parra et al., 1990; Ohlinger *et al.*, 1990]. The RHD virus (RHDV) capsid protein (VP60) folds in two

N-terminal 1 – 234 residues constitute the inner domain and the C-terminal residues beyond 235–579 constitute the protruding domain. In the overall picture of the capsid, these domains form the inner shell and the outer shell respectively, which are characterised by arch-like structures [Barcena *et al.*, 2004] (Figure 2). This structure also correlates with the antigenic characteristics of RHDV, in fact the main antigenic determinants are located on the C-terminal end of the VP60 [Capucci *et al.*, 1995; Capucci *et al.*, 1998; Schirrmaier *et al.*, 1999; Wirblich *et al.*, 1994] (Figure 3).

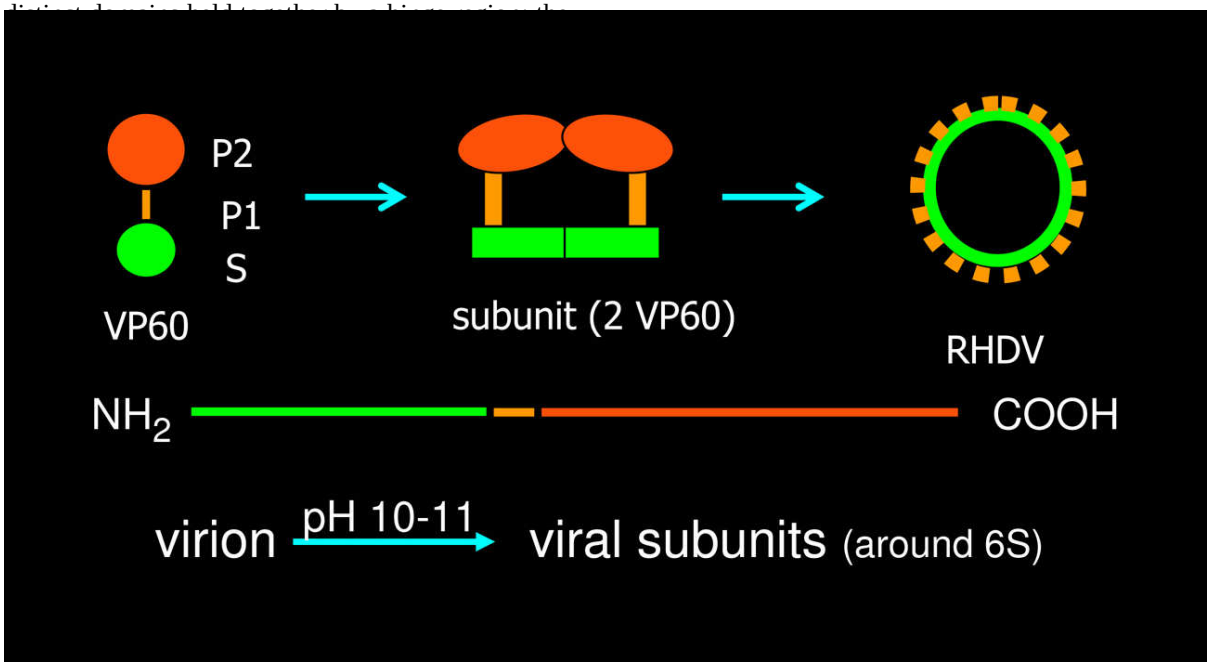

Figure 2. *Schematic representation of the folding of the (VP60) capsid protein of RHDV*

Table 1. Main characteristics of smooth RHDV (sRHDV) in comparison with "full" mature RHD virions

	RHDV	sRHDV
DIAMETER (nm)	32-35	25-30
SEDIMENTATION (S)	170	145
STRUCT. PROTEIN (Kd)	60	28-30
HA (extract 10%)	4-8x103	NEG
INFECTIVITY (LD_{50}) (1 ml extract 10%)	105-107	? NEG ?
ANTIGENICITY		
- RHDV MAbs (ext. epitopes)	pos	neg
- RHDV MAbs (int. epitopes)	pos	pos
- EBHS MAbs (ext. epitopes)	neg	pos
- □RHDV serum	pos	pos
- □EBHSV serum	neg	pos

A second type of virus particle is commonly found as the main component in approximately 5% of the RHDV-positive specimens, i.e. those taken from rabbits showing a protracted course of the disease [Barbieri *et al.*, 1997; Capucci *et al.*, 1991; Granzow *et al.*, 1996]. The main characteristics of this particle, called "smooth RHDV" (s-RHDV) are reported in Table 1. It corresponds to the inner shell of RHDV and large amounts of it could be detected especially from 3–4 days post-infection, when specific anti-RHDV IgM are appearing, only in the liver and spleen and not in the

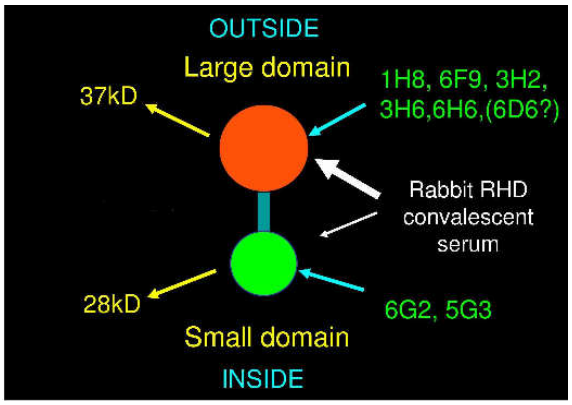

Figure 3. *Schematic representation of the VP60 structure and antigenicity according to the study of Capucci et al., (1995).*

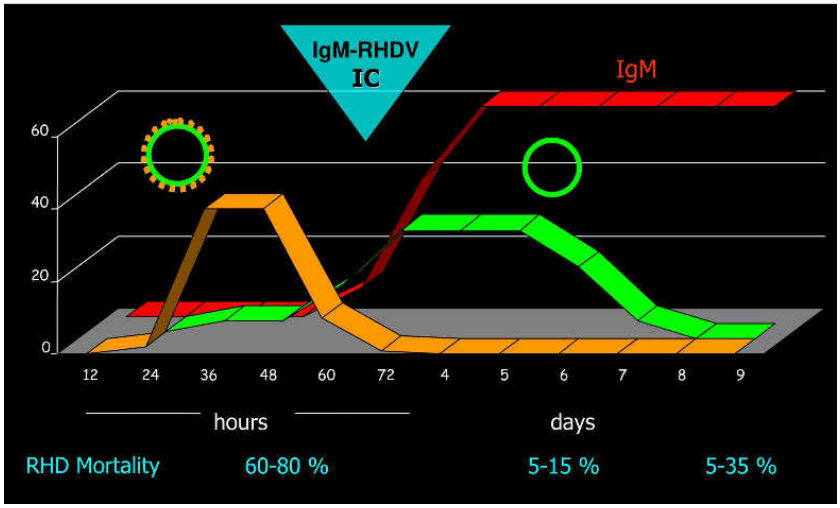

Figure 4. *Timing and evolution of RHD infection following i.m. inoculation of rabbits with a virulent RHDV strain.*

bloodstream These data, in association with the finding of fragments of the VP60 having different molecular weight (41–30 kDa), during transition from RHDV to s-RHDV led Barbieri *et al.* [1997] to conclude that the genesis of the particle is due to a degradative process that is probably the consequence of the physiological clearance of the RHDV-IgM immuno-complex formed in large amounts at the beginning of the humeral response (Figure 4). Therefore the identification of this second particle in the liver of a rabbit can be considered to be a marker of the sub acute/chronic form of RHD that usually evolves between 4 and 8 days post-infection and is followed either by the death of the rabbit or, more often, by its recovery [Barbieri *et al.*, 1997].

RHDV is very stable and resistant in the environment; the viral infectivity is not reduced by treatment with ether or chloroform and trypsin, by exposure to pH 3.0, or by heating to 50°C for 1 hour (Capucci, unpublished data). RHDV in rabbit carcasses can survive for at least 3 month in the field, while virus exposed directly to environmental conditions is viable for a period less than a month [Henning *et al.*, 2005]. Indeed, according to Smid *et al.* [1991] the virus survives at least 225 days in an organ suspension kept at 4°C, at least 105 days in the dried state on cloth at room temperature, and at least 2 days at 60°C, both in organ suspension and in the dried state.

Treatment of RHD virions at pH 11 induces the breakdown of the virions and the production of 6S VP60 subunits (Capucci, unpublished data). RHDV is inactivated by 10% sodium hydroxide, by 1.0–1.4% formaldehyde and by 0.2–0.5% beta-propiolactone at 4°C. Such treatments do not alter the immunogenicity of the virus.

3. Virus variability

All known RHD viral isolates belong to one serotype. The complete sequence of geographically different RHD strains has been reported and their comparison reveals close overall homology in terms of genome sequence with few or no predicted changes in amino acid composition (differences between 2% and 5%) [Le Gall *et al.*, 1998; Nowotny *et al.*, 1997]. Nevertheless, isolates that exhibit temperature-dependant differences in haemoagglutinating characteristics [Capucci *et al.*, 1996a] have been described, and a consistent genetic and antigenic RHDV variant has been identified simultaneously in Italy [Capucci *et al.*, 1998] and Germany [Schirrmaier *et al.*, 1999]. This RHDV variant, named RHDVa, presents amino acid changes in the surface-exposed E region (aa 344–434) that contains the main antigenic epitopes of calicivirus, three times higher than in all previously sequenced RHDV isolates (Figure 5). However, rabbits experimentally vaccinated with the currently available RHDV vaccine were protected from the challenge with RHDVa, even with a lower efficiency.

An epidemiological study carried out to compare the rate of diffusion of RHDV and RHDVa in Italy during the last years [Lavazza *et al.*, 2004] has shown that RHDVa is present in most parts of Italy and that it is rapidly replacing the RHDV "classical" strain (Table 2). Outside Italy, RHDVa was identified almost contemporaneously in Germany but it also caused the first outbreaks of RHD in USA in spring 2000, in Uruguay in winter 2004 and again in USA on 2005. It has also been detected in France (2000) and Malta (2004), which suggests that RHDVa could be diffused in other European countries that have been experiencing the disease for many years. Finally, looking at the

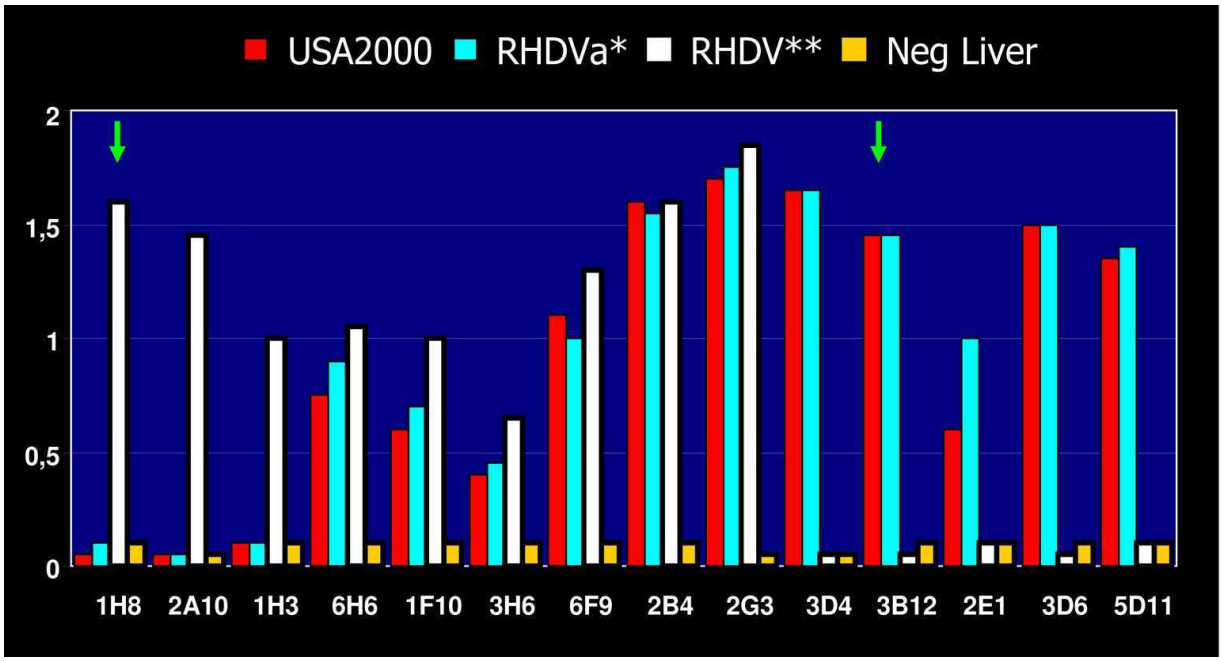

Figure 5. *RHDV antigenic typing using MAbs: the first strain of RHDVa identified at Pavia on 1997 (*) is compared with the RHDV classical strain BS89 (**) and one RHDVa strain isolated in USA on 2000. The arrow indicates the two most relevant MAbs used for differentiating the "classical" RHD from the "variant" RHDVa strain..*

Table 2. Total number of RHD cases observed in Italy during the last four years and relative frequency of classical (RHDV) and Variant (RHDVa) strains.

Year	Total samples examined.	Total RHD positive (%)	(%)RHDV-Positives	(%)RHDVa. Positives
2000	252	134 (53,2%)	89 (66,4%)	45 (33,6%)
2001	136	69 (50,6%)	25 (36,2%)	44 (63,8%)
2002	203	138 (67,9%)	61 (44,2%)	77 (55,8%)
2003	226	63 (25,9%)	12 (19,0%)	51 (81,0%)
2004	209	124 (59,3%)	32 (25,8%)	92 (74,2%)

RHDV genetic sequences deposited at the NCBI databank, the presence of RHDVa in China is evident too.

Another virus, provisionally called rabbit calicivirus (RCV) and related to the RHDV, has been identified in healthy rabbits [Capucci *et al.*, 1996b; 1997]. It is significantly different from the previously characterised RHDV isolates in terms of pathogenicity, viral titre and tissue tropism. RCV is avirulent, replicates in the intestine at a very low titre and has about a 92% genomic similarity to RHDV from which follows a high degree of antigenic correlations.

Recent studies conducted in Italy have shown that such virus is quite widespread in industrial rabbit farms [Capucci *et al.*, 2004b]. In fact, in order to check the diffusion of RCV in Italian rabbit farms we conducted, along a 5 years period: (1999-2004), a survey respectively in 39 farms in North Italy, 23 farms in Central Italy and 21 farms in South Italy, by testing non-vaccinated 80 day old growing rabbits at the slaughterhouse. The results indicate the presence of "natural antibodies" presumably induced by RCV, i.e. over 75% of animals showing titres $\geq$1/20, in almost 30% of farms controlled in North and South Italy, and in 52.2% of the farms controlled in Central Italy (Table 3).

As result of the extensive use of serological test on different rabbits populations, further evidence exist that, in addition to RCV, one or more RHDV-like non-pathogenic viruses are present in wild rabbit populations in a large part of south-eastern Australia as well as in New Zealand [Cooke *et al.*, 2002; Nagesha *et al.*, 2000; O'Keefe *et al.*, 1999; Robinson *et al.*, 2002]. The serological data indicate that the putative RHDV-like virus suspected to be present in Oceania is characterized, differently than

Table 3. Results of seroepidemiological surveys for detecting anti-RCV antibodies in non-vaccinated grow slaughterhouse.

Serological result	Criteria applied	N. groups tested (%)		
		North Italy 1999	Central- South Italy 2002-03	Central Italy 2004
Positive	> 75% of positive sera	13 (33,3%)	4 (19,1%)	12 (52,2%)
Doubtful	5-10% of positive sera	2 (5,2%)	0 (0%)	2 (8,7%)
	20-60% of positive sera	0 (0%)	5 (23,8%)	2 (8,7%)
Seroconversion	from 0% to >75% of positive sera	0 (0%)	1 (4,7%)	0 (0%)
Negative	> 95% of positive sera	24 (61,5%)	11 (52,4%)	7 (30,4%)
Total		39	21	23

RCV, by a consistent genetic and antigenic difference from RHDV, estimable in more than 40% of amino acid substitution in the outer part of the VP60 [Capucci personal observations].
Antibodies against RHD were detected in sera collected in Europe between 1975 and 1987, showing that RHDV-like viruses were already present, but simply had not been detected before the first evidence of the disease [Rodak *et al.*, 1990]. More recent serological data suggest that non-pathogenic strains may usually be present in wild European rabbit populations, because high antibody levels have been detected even where RHD had never been recorded or suspected [Marchendeau *et al.*, 2005].

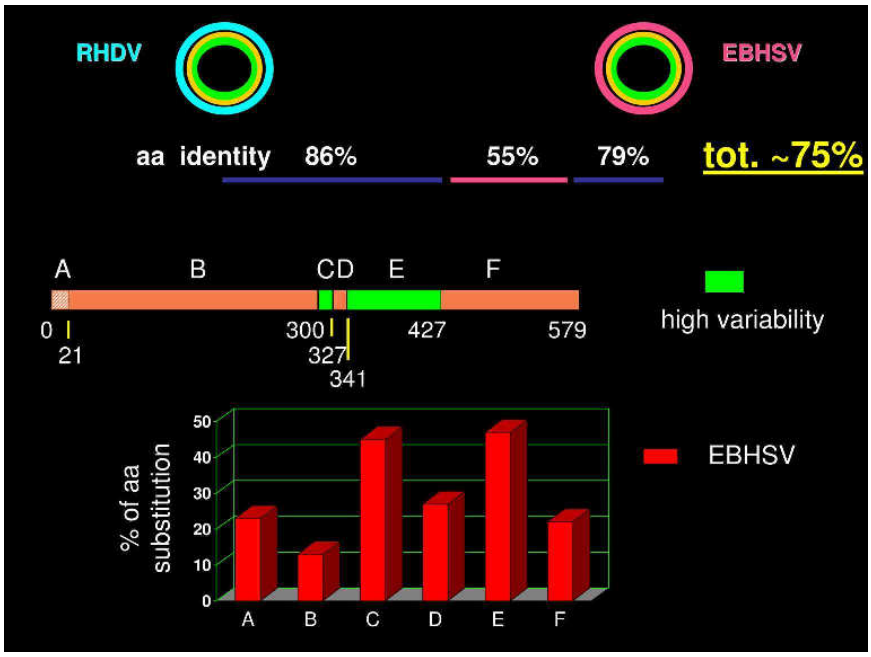

Figure 6. *Schematic representation of the structural differences between RHDV and EBHSV. The subdivision of the structural protein of RHDV in relation to the degree of variability in Calicivirus was done according to Neill (1992).*

4. The disease

The European rabbit (*Oryctolagus cuniculus*) is the only species affected by RHD and no other lagomorphs of the genus *Romerolagus*, *Lepus* and *Sylvilagus* (including the cottontail) normally present in North Central and South America have been shown to be susceptible [Gould *et al.*, 1997]. A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (*Lepus europaeus*), but the causative calicivirus is different from RHDV, although it is related antigenically [Capucci *et al.*, 1991] (Figure 6). Cross infection does not occur by experimental infection of rabbits with EBHSV and hares with RHDV [Lavazza *et al.*, 1996]. Recent studies aimed at finding the susceptibility of cottontail to EBHSV revealed a diffuse seroprevalence of the virus in a wild population of cottontail rabbits and the possibility of inducing clinical disease and mortality in a low number of experimentally infected cottontails [Tizzani *et al.*, 2002]. RHD is characterised by high morbidity and a mortality rate between 40%

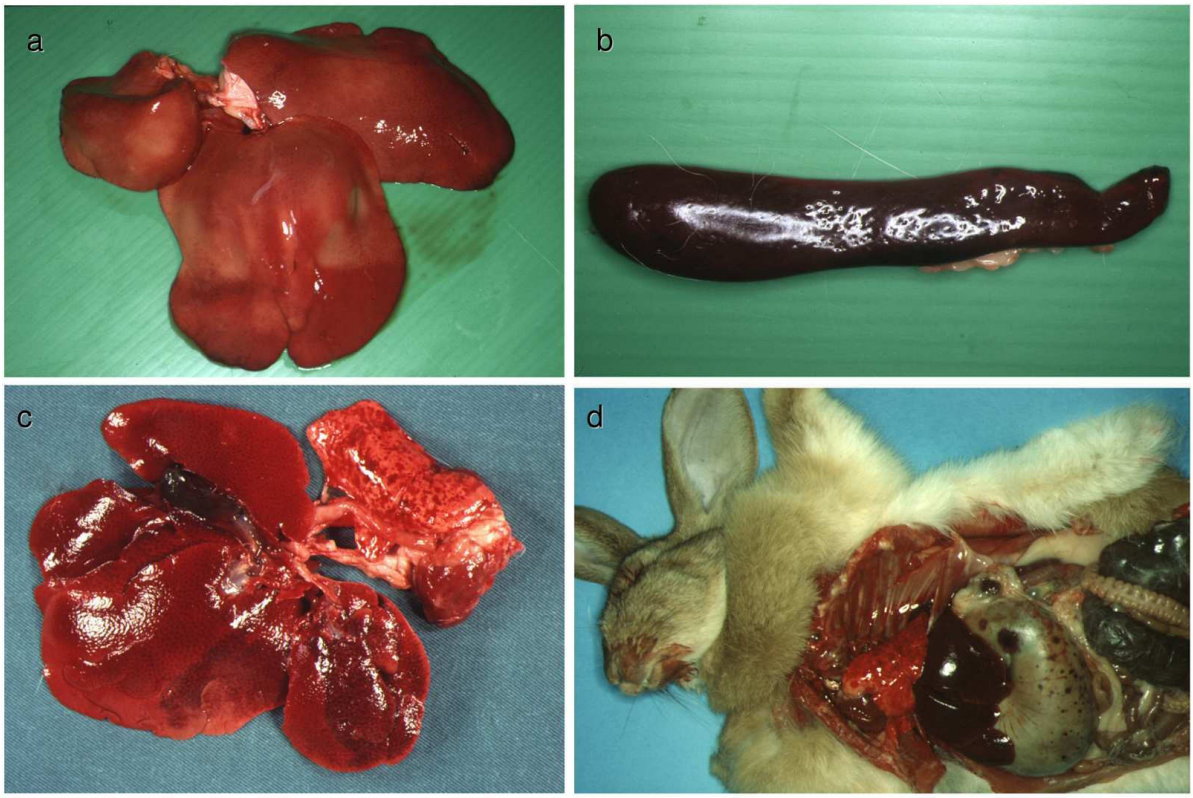

Figure 7. *RHDV macroscopic lesions: a) liver degeneration: the liver is enlarged, discoloured and friable. b) spleen enlargement and congestion. c) liver congestion and lung haemorrhages. d) rabbit die due to acute acute disease shows diffuse haemorrhages and a sero-heamorrhagic liquid from the nostrils*

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and 90%. Infection occurs in rabbits of all ages, but clinical disease is observed only in adults and young animals older than 40–50 days. The pathogenic mechanism of resistance in young animals is still unclear [Cooke, 2002]. A difference in the cellular inflammatory response of the liver following an RHDV infection of susceptible adult rabbits and resistant young ones was observed, and the persistence, following RHDV infection in young rabbits, of increased value of liver transaminases determines a chronic course of the disease and the possible role of these animals as a source of virus transmission [Ferreira *et al.*, 2004; 2005].

The clinical evolution of the disease [Marcato *et al.*, 1991] can be peracute/acute and subacute/chronic. The acute disease is characterized by few signs and sudden mortality (nervous signs in agonic phase, dyspnoea and even mortality within 48-96 hrs The incubation period varies between 1 and 3 days; death may occur 12–36 hours after the onset of fever (>40°C). During an outbreak, a limited number of rabbits (5–10%) may show a subacute/chronic or even a subclinical evolution of the disease. These animals often die 1 or 2 weeks later, probably due to a liver dysfunction (Figure 4).

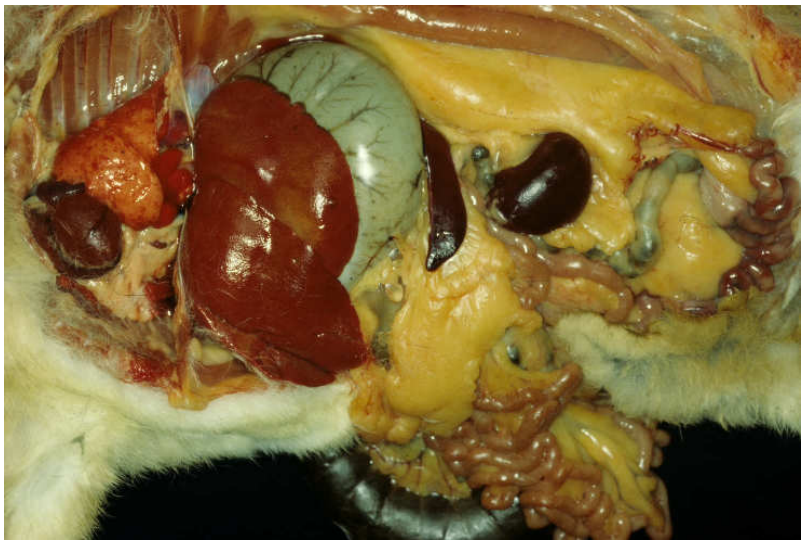

Figure 8. *Rabbit die do to subacute-chronic disease shows liver degeneration and an icteric discoloration of the visceral fat and subcutis.*

The gross pathological lesions [Marcato *et al.*, 1991] are variable and may be subtle. Liver necrosis and splenomegaly are the primary lesions (Figure 7a, b,) However, a

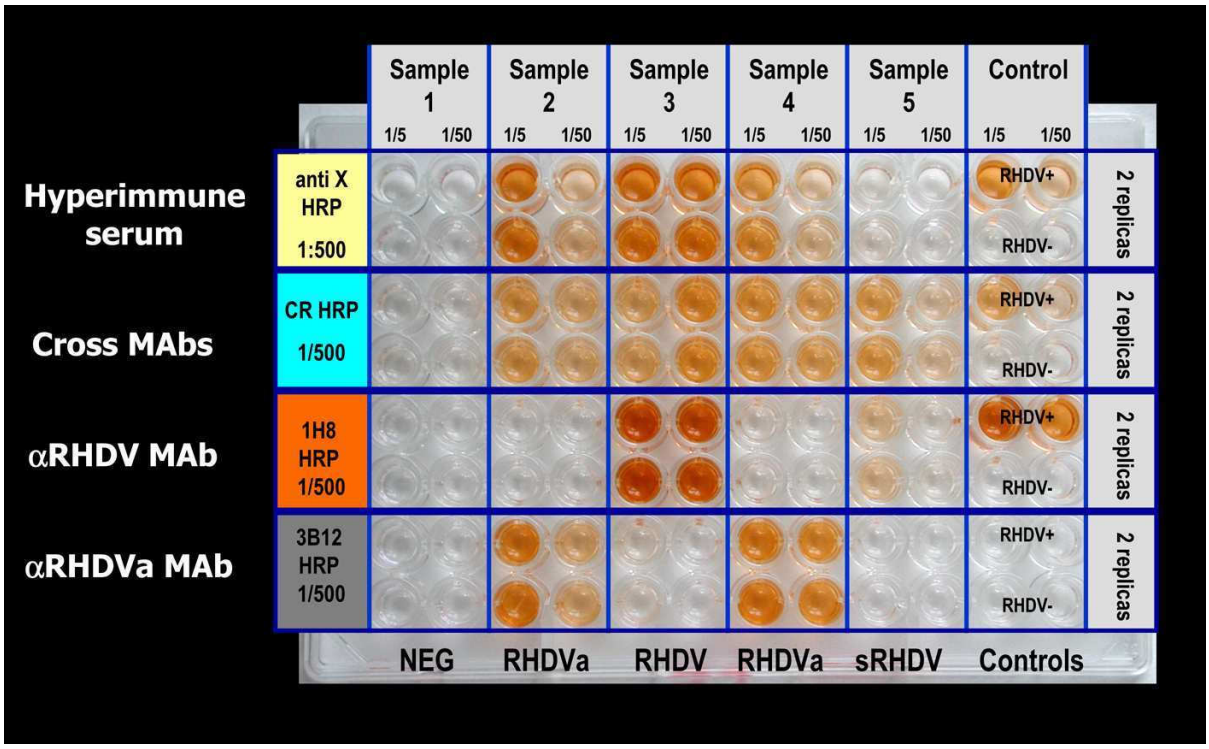

Figure 9. *ELISA test for RHDV routine diagnosis using RHDV and RHDVa specific MAbs: sample 1 is negative, samples 2 and 4 are RHDVa variant, sample 3 is a "classical" RHDV and sample 5 is a smooth "degraded" RHDV.*

massive coagulopathy is usually the cause of haemorrhages in a variety of organs and sudden death (Figure 7c,d). In subacute and chronic disease, an icteric discoloration on the ears, conjunctiva and subcutis is clearly evident (Figure. 8).

5. Diagnosis

Presumptive diagnosis is based on clinical signs, lesions and epidemiology (respiratory distress, high mortality and rapid spread); diagnosis of confirmation as well as strain characterization is achieved by laboratory tests.

The liver contains the highest viral titer and is the organ of choice to submit to viral identification. The amount of virus present in other parts of the body is directly proportional to vascularization; thus spleen, lungs and serum are quite rich in virus and can serve as alternative diagnostic material. Tissue suspensions of organs (5-20% w/v) can be directly examined by hemagglutination (HA) test using human type O erythrocytes, electron microscopy or enzyme-linked immunosorbent assay (ELISA).

The test commonly used for routine examinations are:

1) Sandwich ELISA using RHDV specific Monoclonal Antibody (MAb) [Capucci *et al.*, 1995; Capucci and Lavazza, 2004] (Figure 9).

2) Sandwich ELISA test using a panel of RHDV specific MAbs. This test permits the identification of RHDV variants and particularly to distinguish between the original RHD virus and its first consistent antigenic variant RHDVa [Capucci *et al.*, 1998].

3) Western Blot analysis using RHDV-MAbs that recognize internal epitopes and also cross-reactive with EBHSV [Capucci *et al.*, 1991]. It is usually performed on the few samples, which give doubtful results in Elisa test, and in animals died due to the "chronic" form of the disease.

Other diagnostic methods have been developed including plate agglutination test, immunostaining of paraffin embedded sections, fluorescent antibody test on tissue cryosections, western blot, *in situ* hybridization. Reverse transcription Polymerase Chain Reaction (RT-PCR) [Guitrre *et al.*, 1995; Gould *et al.*, 1997] is an extremely sensitive method for the detection of RHDV and it is 10^4-fold more sensitive than ELISA. However RT-PCR is not strictly necessary for routine diagnosis but it is more appropriate for investigations on molecular epidemiology, to study the pathogenesis of the infection and to detect virions in young animals at the time they get infected and are not diseased (less than 40-50 days of age), in non-specific hosts (other vertebrates) and in vectors (mosquitoes and fleas).

As no satisfactory growth condition and sensitive cell substrates have been established, *in vitro* isolation of RHD virus cannot be included among the virological methods. Therefore, to date viral isolation *in vivo* by experimental reproduction of RHD retains paramount importance. In fact large quantities of viral antigen are needed to prepare diagnostic reagents and produce inactivated tissue-

derived vaccines. Experimental infection is not practical as a routine diagnostic method although it is still desirable in the case of unusual samples (HA negative / ELISA positive) or not clearly positive.

To succeed in reproducing the disease, the inoculated rabbits must be fully susceptible to the virus. Susceptibility depends both on the age of animals, which should be more than two months old, and on the absence of specific antibodies, even at low titres.

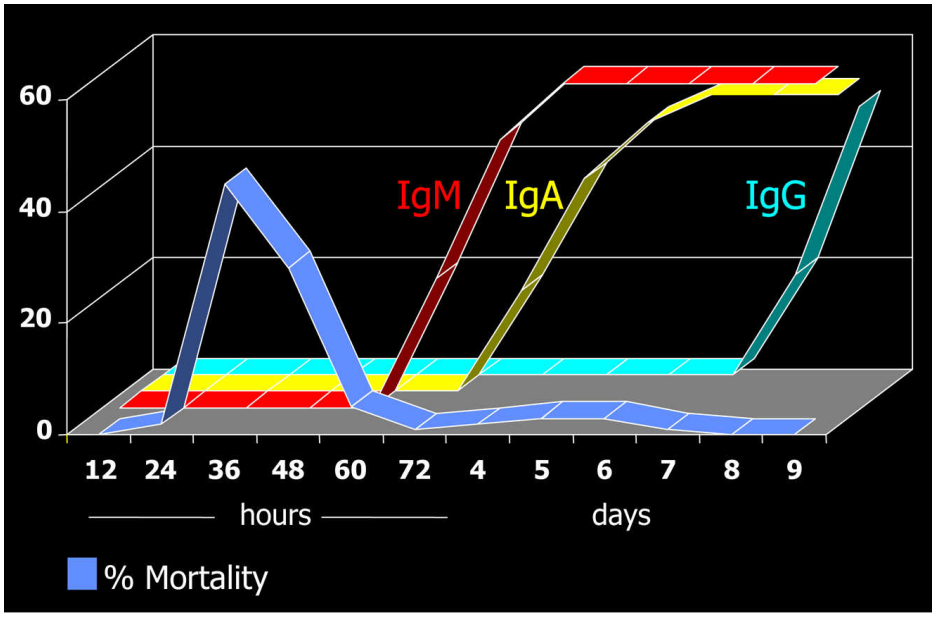

Figure 10. *Schematic representation of the humoral response in rabbits following i.m. inoculation of a virulent RHDV strain, compared with mortality rate.*

Infection by RHDV can be diagnosed through detection of a specific antibody response. Animals that overcome the disease present a striking seroconversion, which can be easily detected 4-6 days p.i. (Figure 10). Indeed, as the humoral response is of great importance in protecting animals from RHD, determination of the specific antibody titer after vaccination or in convalescent animals is predictive of the ability of rabbits to resist RHD virus infection.

Three basic techniques are applied to the serological diagnosis of RHDV: haemoagglutination inhibition (HI) [Gregg, 1992], indirect ELISA and competitive ELISA [Capucci *et al.*, 1991; Capucci *et al.*, 2004a]. With respect to the availability of reagents and technical complexity HI is certainly the most convenient method. On the other hand ELISA reactions are more easily and quickly performed, particularly when a high number of samples are tested. The sensitivity and specificity of competition ELISA (cELISA) using MAbs is markedly higher than those achievable with other methods [Capucci *et al.*, 1991] since it mainly measures antibodies directed against antigenic determinants on the external surface of the virus, usually the most specific and functionally important. Therefore it is considered the standard and reference test for RHD.

Three additional sandwich ELISA tests were developed using antisotype MAbs (isoELISAs) to test the sera for the presence of specific anti-RHDV IgM, IgA and IgG. The isotype titres could be critical for the interpretation of field serology and for correctly classifying the immunological status of rabbits [Cooke *et al.*, 2000]. Some other tests could be used for specific investigations and particularly when a higher level of sensitivity is needed in order to detect antibodies in non-target species (including humans) or antibodies induced by cross reacting RHDV-like agents. They include: 1) Indirect ELISA (inELISA); it has a slight higher sensitivity in respect to cELISA, making possible to measure highly cross-reactive antibodies, and it can detect antibodies with low avidity. 2) Solid phase ELISA (spELISA); the purified antigen is directly adsorbed to the solid phase and due to virus deformation internal epitopes are exposed. Therefore it detects a wider spectrum of antibodies with high sensitivity and low specificity. 3) Sandwich Elisa to detect IgM and IgG in liver or spleen samples already examined with the virological test. Such test is particularly useful in those animals which die due to the "chronic" form of the disease, when the detection of the virus could be difficult. In this case, a high level of RHDV specific IgM and a low level, if any, of IgG are the unambiguous marker of RHD positive samples.

Technical details and full references on the different virological and serological tests are reported in the RHDV dedicated chapter in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [Capucci and Lavazza, 2004a].

6. Epidemiology. Exposure factors. High and Low risk assessment

Incidence of RHDV in industrial units is low since the disease can be easily controlled by vaccination. In the recent year the spreading of the new variant strain (RHDVa) has determined an increase of outbreaks due to vaccination failures [Lavazza *et al.*, 2004]

Currently RHD is endemic in East Asia, Europe and in Australia and New Zealand. Outbreaks have also been recorded in Central America (Mexico and

Cuba), Saudi Arabia and West and North Africa. In 2000 and 2001 three independent outbreaks were recorded in the United States of America. The endemic persistence of RHD in a country is usually guaranteed by the spreading of the disease in rural units and wild animals.

RHD spreads very rapidly and infection can occur by nasal, conjunctival or oral routes. The disease is commonly observed throughout the year and could be transmitted directly or indirectly by equipments, cages, instruments, humans, birds, insects etc. [Allegranza, *et al.*, 1990; Asgari *et al.*, 1998; Cooke, 2002]. Indirect transmission by means of animated vectors, including man, or unanimated tools is favored by the high stability and resistance of the virus in the environment. Wild rabbit population can act as reservoir. Among the risk factors that should be considered for explaining the occurrence of outbreaks in industrial farms are: 1) the introduction of breeders of unknown origin and/or without application of quarantine period; 2) the transport of animals when trucks visit farms to pick up animals to go to the abattoir.

7. Prophylaxis - Good agricultural practices

Where RHD is endemic, an indirect control of the disease in industrial rabbitries is mainly achieved by vaccination. Indeed, the application of strict biosecurity measures is suggested to prevent the introduction of the infection in industrial farms. Some sanitary and environmental arrangements are very helpful, including: 1) the application of biosecurity programs; 2) the culling and removal of ill or dead animals; 3) the cleaning and disinfection of equipment, cages, instruments etc.; 4) the use of single use instruments for AI and therapies; 5) visitor controls: restriction to visits of humans and other animals such as dogs and cats; 6) insect traps at the windows and ventilation intakes; 7) avoiding wild rabbits entering the farm.

Vaccination is a routine practice in industrial rabbit farm. Vaccines are usually prepared by using clarified liver suspension of experimentally infected rabbits, subsequently inactivated and adjuvated (see more details in the RHDV chapter in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [Capucci and Lavazza, 2004a]). Vaccinated breeders quickly produce stray humeral immunity i.e. within 10-15 days post vaccination. The usual program is to administer the inactivated vaccine twice with an interval of at least two weeks.

Normally, a 1 ml dose is inoculated subcutaneously in the neck region. In those units where the anamnesis for RHD is negative, it is advisable to vaccinate only the breeding stocks; the first injection should be done at three months of age. Annual re-vaccination is strongly recommended to ensure a good level of protection, although experimental data indicate that protection usually lasts for a long time (more than one year) [Arguello-Villares, 1991].

Growing rabbits are usually not vaccinated if the sanitary situation of the farm is normal, since their susceptibility period is quite narrow i.e. between 35-40 days of age to slaughtering age around 80 days. Nevertheless in area at risk or after major outbreak, even if strict hygienic and sanitary measures are adopted, it is strongly recommended to vaccinate growing rabbits at the age of 40 days because the incidence of infection/re-infection is very high. Only after a certain number of production cycles it is advisable to stop vaccination and to do so a variable number of growing rabbits, starting with a small group, should not be vaccinated in order to verify the persistence of infective RHD inside the unit.

Vaccination could also be considered a quite effective post-exposure treatment to be included in the emergency strategies applied when RHD occurs in rabbitries. Indeed, better results in limiting the diffusion of the disease and reducing the economic losses could be obtained by using seroterapy through the parenteral administration of anti-RHDV hyperimmune sera.

Other types of vaccines based on biotechnologies have been prepared and experimented with, with some equally good results but none of them is presently commercially available [Capucci and Lavazza, 2004a].

8. Conclusions

Due to the broad antigenic and genomic variability of rabbit caliciviruses the importance of a continuous epidemiological and antigenic surveillance on RHD must be stressed, also considering that an efficacious vaccine is the main, if not the only, tool to protect rabbits. Indeed, the combination of the available different serological and virological methods of diagnosis provides novel and highly sensitive means for the identification and characterisation of such viruses, with special regard to genome composition, evolution, features of pathogenicity and molecular epizootiology.

Nevertheless, the complex epidemiological pattern of RHD should consider the potential role of non-pathogenic strains of RHDV-like viruses, also potentially derived by the attenuation of the original RHDV strains, and, therefore it is particularly important that serological surveys are made using methods able to distinguish between antibodies that are protective against RHD and antibodies that are not. At the same it must be a priority for future research to isolate and characterize the RHDV-like strains in order to determine the level of protection that each of them can induce and to better understand the epidemiology of RHD in wild as well as domestic and industrial populations.

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Chapter 4

NUTRITION AND FEEDING STRATEGIES FOR IMPROVING THE HEALTH OF THE DOE AND THE YOUNG RABBIT

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Specific or non-specific enteropathy remain a major problem for the growing rabbit (about 15 to 25% from birth to slaughter), while the nutrition of the doe and its corporal status is a crucial point implicated in the reproductive performances, health and career length. However, studies that deal with the interactions between nutrition and health are not numerous, although this is one of the priorities in European rabbit breeding systems. Indeed, studying this topic needs large numbers of animals bred in controlled experimental facilities.

For instance, two French networks of experimentation are studying feeding and nutritional factors implicated respectively in the health of the weaned rabbit (GEC group) or doe and sucking young (GERC group). Network of European researchers could help in solving this problem, and a research group on nutrition and pathology was created inside the action COST848. Research was done in several ways, either in a therapeutic approach, or in a preventive approach to improve the digestive health of the young (see subchapter 4.3) or the nutrition of the doe (see subchapter 4.4).

However, the knowledge of nutritional needs of rabbits requires a more complete understanding of its digestive physiology, including the gut microbial ecosystem (see subchapters 4.1 and 4.2). In addition, an adequate feeding strategy should be found for the period around weaning to cover the different needs of both mother and litter (see subchapters 4.4 and 4.5), since they receive the same feed in the current breeding systems.

The interaction between nutrition and digestive pathology has been mainly explored for the growing rabbit after weaning. The nutritional preparation of the young before weaning is probably a key step determining the digestive health of the growing rabbit. This should provide new concepts for the nutrition of the young between 3 and 5 weeks old.

With respect to the nutrition of the doe, short and especially long term criteria (such body management, career length) should be considered in the future. Interactions between the nutrition of the young should also be considered in the research programs. The availability of this information will allow the development of global nutritional strategies for reproductive rabbit does and their litter. However, global nutritional strategies taking into account the productivity of the reproductive doe in the long term (body condition, health and longevity) and the possible effect on farm health should be studied on a larger scale, implicating alternatives strategies reducing the use of drugs in breeding.In subchapter 4.6, the interest of several feed "additives" which can offer alternatives to the use of in-feed antibiotics are reviewed.

4.1. Recent advances in the digestive physiology of the growing rabbit

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1. Introduction

Digestive disorders are essentially encountered in the growing rabbit, and not in the adult. Thus, several researchers studied the relationship between digestive physiology (including feeding behaviour) of the young and the consequence on digestive health. This subchapter aims at decribing recent knowledge in the digestive physiology of the young rabbit from 2 to 6 weeks of age, since it is a key period framing the weaning and where the maturation of the digestive functions is very active. Moreover, the digestive maturation according to several breeding factors, such as age at weaning or feeding restriction, is described.

2. Elements of digestive anatomy and feeding behaviour in the rabbit, including caecotrophy.

Only some basis of digestive physiology and feeding behaviour is given here for the domestic rabbit bred in standard commercial systems. More detailed information is available in recent reviews (Lebas *et al.*, 1997; Carabaño *et al.*, 1998; Gidenne and Fortun-Lamothe, 2002; Gidenne and Lebas, 2005).

2.1. Digestive anatomy and caecotrophy

The digestive system of the rabbit is adapted to an herbivorous diet, including specific adaptations, from teeth to an enlarged caeco-colic segment with active microbiota, and the separation of caecal digesta particles allowing for caecotrophy. The general organisation of the digestive tract is presented in Figure 1.

Contrary to other mammals, the pH of the rabbit stomach is always very acid (1.5 to 2.0), and varies along the day mainly in the fundus in relation with soft faeces presence. Glands included in the stomach wall secrete hydrochloric acid, pepsin and some ions (Ca^{++}, K^{+}, Mg^{++} and Na^{+}). The small intestine works similarly to other monogastric mammals, the digesta are liquid (8-10% DM), especially in the upper part. Their pH is slightly basic in the upper part (7.2-7.5) and more acid in the end of ileum (6.2 - 6.5). The caecum is the largest segment of the tract (40% of the whole digestive content), and contains 100 to 120 g of a uniform pasty mix (21-24% DM).

The caecal pH varies around 6.0 depending on microbial activity and feeding pattern. The functioning of the rabbit's upper digestive tract is globally the same as that of other monogastric domestic mammals. Specificity of rabbit (and of *Lagomorpha* in general) lies in the dual function of the proximal colon. If the caecum contents enter the colon in the early part of the morning they undergo few biochemical changes. The colon wall secretes mucus, which gradually envelops the pellets formed by the wall contractions. These pellets gather in elongated clusters and are called soft faeces (more scientifically, caecotrophes).

If the caecal digesta enter the colon at another time of the day, the activity of the proximal colon is entirely different. Successive waves of contractions in alternating directions begin to act; the first to evacuate the contents normally and the second to push them back into the caecum. Most of the liquid fraction (soluble products and small particles < to 0.1 mm) is forced back into the caecum (Björnhag, 1972). The solid part, containing mainly large

particles over 0.3 mm long, forms hard faeces. This dual action of the colon produces two types of faeces: hard and soft, the later is richer in protein (half of bacterial origin) and water-vitamins (B and C). While hard faeces are excreted, the soft ones are ingested by the rabbit directly upon being expelled from the anus.

Caecotrophy must not be confounded with coprophagy (when animals have only one type of excrement), since it is an herbivorous strategy to benefit from the microbial protein. Indeed, soft faeces represent three quarters of the total stomach contents at the end of the morning.

The particular functioning of the colon requires roughage feeds. If the feed contains a high proportion of small particles (<0.1mm), most of the caecal contents are pushed back to the caecum, and serve as "nutrients" to the caecal microbial flora.

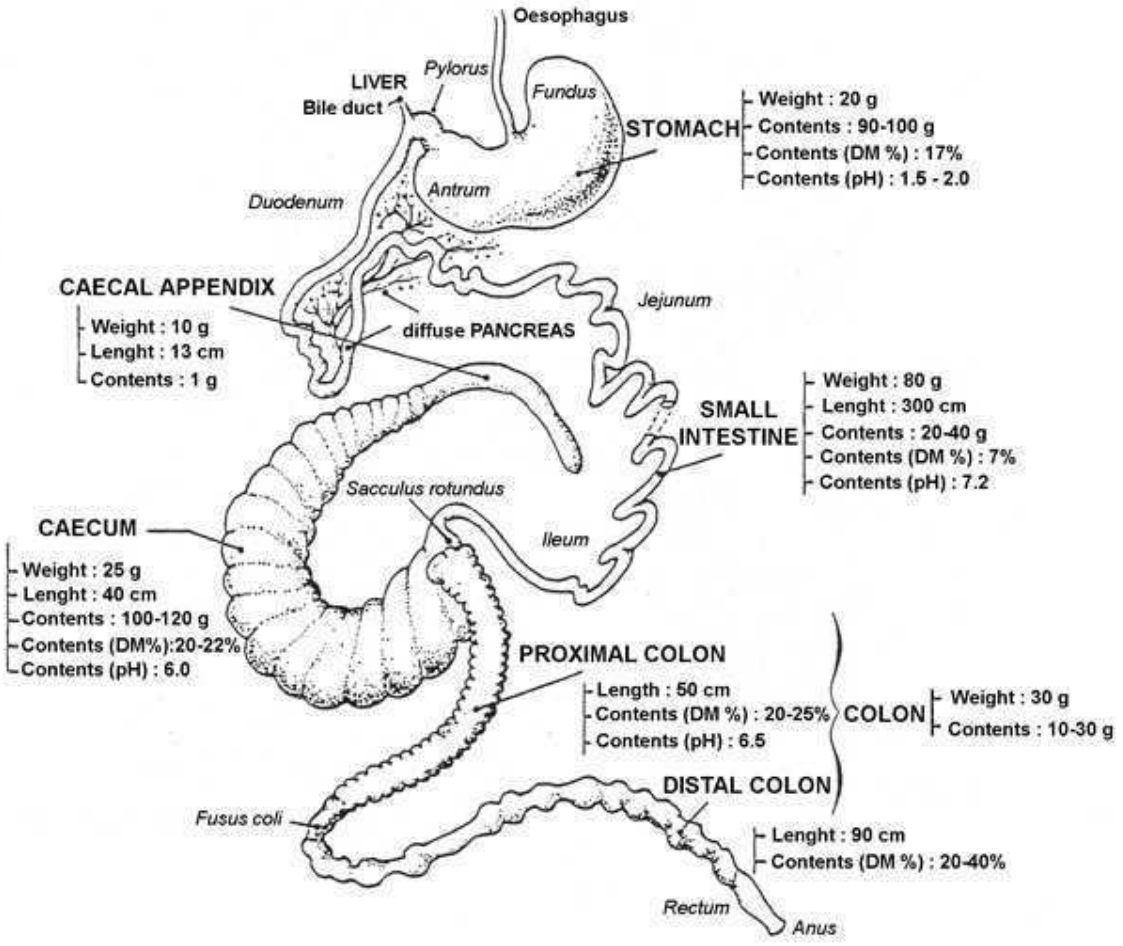
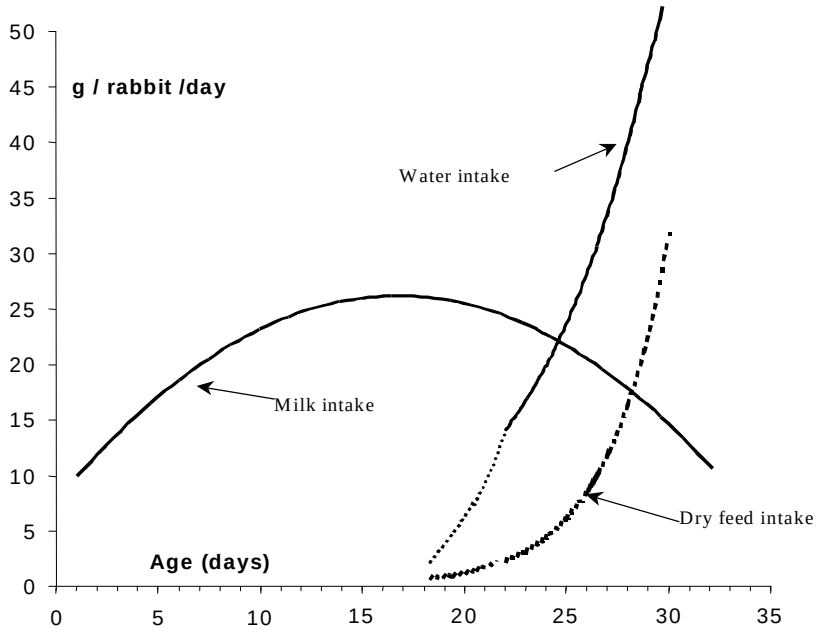


Figure 1. *The digestive tract of a rabbit. Numerical values are those observed in a 2.5 kg New Zealand White rabbit, fed ad-lib. a pelleted balanced diet (according to Lebas et al., 1997).*

2.2. Feeding behaviour

The rabbit feeding behaviour is very peculiar compared to other mammals, with special features, such as caecotrophy, associated with a particular digestive physiology, intermediate between the monogastric and the herbivore. **From birth to weaning,** the doe suckles her litter mainly once a day. Suckling lasts only 2-3 minutes for a litter of 8 to 11 kits. Reversely, kits are able to suckle twice a day or more, and from two different does within a day, leading to a higher growth rate (Gyarmati *et al.*, 2000a). From one to three weeks of age, the young increases its milk intake from 10 to 30 g /day (Figure 2). Then the doe milk production decreases, more sharply if the doe is in late pregnancy. During the first post-natal week (between 4 and 6 days of age) the young also consumes hard faeces deposited by the doe in the nest, which possibly stimulates the caecal flora maturation (see subchapter 4.2). A young rabbit, reared in a litter of 7-9 kits, therefore consumes about 360 to 450g of milk between birth and 25 d old (100 to 150g from 26 to 32d). Individual milk intake patterns are relatively variable and dependent partly on the live-weight of the kit.



Mean values, for litters of 7-9 kits, with pelleted dry feed, nipple drinker, weaned at 30d, and with doe remated 11d after kindling (from Gidenne and Lebas, 2006)

Figure 2. *Milk, water and solid feed intake of the young rabbit, before weaning*

The dry feed intake becomes significant when the young is able to easily access a feeder (with pelleted feed) and a drinker, i.e. around 17-20 days old (Figure 2). Under classical breeding conditions, the total dry feed intake is about 25-30g per animal for the period 16 to 25d old. Then, the food intake increases 25 fold from 20 to 35 days of age (Gidenne and Fortun-Lamothe, 2002). However, large variations among litters have been observed for the time of the beginning of the solid feed intake. For example, an increase in the competition for milk, dependent on the litter size, stimulates the solid feed intake of the young (Fortun-Lamothe and Gidenne, 2000). Reversely, offering a second suckling to the young (using a second doe) delayed the dry feed intake (Gyarmati *et al.*, 2000). Age at weaning is obviously an efficient factor to modulate the dry feed intake as shown by several studies (Xiccato *et al.*, 2000; Gallois *et al.*, 2005). For instance, in wild condition the young rabbit could be weaned at about 3 weeks of age, when the doe is pregnant and preparing a new nest for her next litter, and thus is forced to consume rapidly solid food.

The period from 25 to 30 days of age is particular, since the intake of solid feed and water exceeds the milk intake. During this period the changes in feeding behaviour are remarkable: the young rabbit goes from a single milk meal per day to a large number of alternating solid and liquid (water) meals irregularly distributed throughout the day. Additionally, the caecotrophy behaviour starts at about 22 to 28 days of age (Orengo and Gidenne 2006), when a significant dry feed intake occurs that leads to a caecal and a colon filling and to the dual motility pattern of the proximal colon. However, the individual feeding behaviour of the young remained largely unknown (regulation factors, number of meals, etc…), since no method is presently available to assess the intake level of young reared collectively (till weaning).

From weaning (4 to 5 weeks of age) the daily feed intake increases correlatively to the metabolic live-weight and levels up at about 5 months of age. Taking as a reference animal an adult fed *ad libitum* (140-150 g DM/day, for example, for a 4 kg New Zealand White): at 4 weeks of age, a young rabbit eats 25% of the amount an adult eats, while its live-weight is only 14% of the adult's. At 8 weeks the relative proportions are 62 and 42%; at 16 weeks they are 100 to 110 and 87%. The rabbit regulates its feed intake according to energy need, as for other mammals. Chemostatic mechanisms are involved, by means of the nervous system and blood levels of compounds used in energy metabolism. However, in monogastric animals the glycemia plays a key role in food intake regulation, while in ruminants the levels of volatile fatty acids in blood have a major role.

Since the rabbit is a monogastric herbivore, the main blood component regulating feed intake is not completely ascertain, but it is likely to be the blood glucose level. Digestible energy (DE) voluntary intake is proportional to metabolic live-weight ($LW^{0.75}$), and is about 900-1000 kJ/d/kg $LW^{0.75}$. The chemostatic regulation appears only with a dietary DE concentration higher than 9-9.5 MJ/kg (Parigi-Bini and Xiccato, 1998). Below this level, a physical-type regulation is prevalent and linked to gut filling. The rabbit fractionates its voluntary solid intake in numerous meals (30 to 40). The number of water drinking evolves in parallel to that of pelleted feed, and the time spent to drink is lower than that spent to eat. Over 60% of the solid feed (excluding soft faeces meals) is consumed in the dark period for a domestic rabbit submitted to a 12L/12D light schedule. The feed intake level is modulated by the physiological status of the animal. For instance, a doe's voluntary intake varies greatly during the reproduction cycle (150 g/d during pregnancy to 400

g/d at the end of the lactation peak). Several external factors are also modulating the feeding behaviour of the domestic rabbit, such the feed composition, or the environmental temperature.

3. Digestive tract maturation: digestion and immune system

The digestive tract has a double function: the digestion of nutrients and the protection from undesirable microorganisms. These two functions maturate gradually after birth, under the influence of ontogenic factors (related to the age and the growth of the individual), diet and interaction between microorganisms, to stabilise themselves between 8 and 10 weeks of age.

To ensure these two functions, the mucosa is composed of the digestive epithelium, the gut-associated lymphoid tissue and the mucus overlying the epithelium (Figure 3).

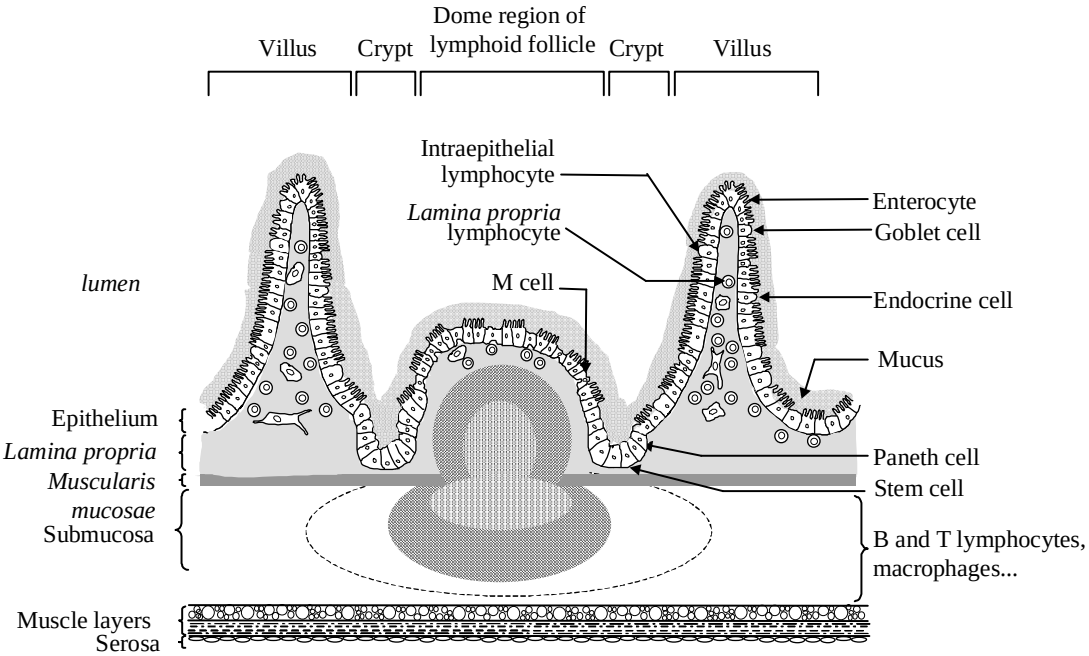


Figure 3. *Organization of the digestive mucosa in rabbit, includig the digestive epithelium (enterocytes, globlets cells and paneth cells) and the gut-associated lymphoid tissue (lymphoid follicles and cells diffused in the lamina propria or in the epithelium).*

3.1. Maturation of the digestive function

The anatomy of the digestive tract is stabilised from 9 weeks of age, except the cæcal appendix which grows until 11 weeks. Between 3 and 11 weeks of age, the weight of organs is multiplied by 4 for stomach and small intestine, by 8 for cæcum and large intestine and by 15 for cæcal appendix. During this period, the growth of the large intestine is higher than that of anterior segments, accounting for 28% of the whole digestive tract at 3 weeks and 44% at 11 weeks. Length is multiplied by 2 to 3 for all of digestive segments between 3 and 11 weeks of age (Lebas and Laplace, 1972; Alus and Edwards, 1977; Xiccato *et al.*, 2001).

During the weeks following birth, morphology of intestinal epithelium deeply changes. Intestinal villa which are thin and lengthened after birth (finger-shaped with a height to width ratio about 3.7-4.0) became broader thereafter (tongue-shaped, near twice longer than wide with height to width ratio around 2.2-2.4; Yu and Chou, 1997; Van der Hage, 1988; Sabatakou *et al.*, 1999). For example, the duodenal villi heightened from 746 µm on day 28 to 940 µm on day 49 of age (Gallois *et al.*, 2005; Figure 4). A proximo-distal decreasing gradient in villus height was apparent starting from day 28. The crypts deepened from day 14 till day 49 (50 µm to 180 µm).Histological maturation of intestinal mucosa is incomplete until day 20 of age and follows a proximo-distal gradient (Toofanian and Targowski, 1982). The caecal and colon mucous walls change from 16 days of age with the appearance of ridges (Yu and Chiou, 1997; Sabatakou *et al.*, 1999a, b), when the intestinal flora establishes together with fermentation activity. The structural modifications that occur during the phase of maturation lead to a very important increase in the exchange surface.

Digestion of nutrients occurs both in stomach

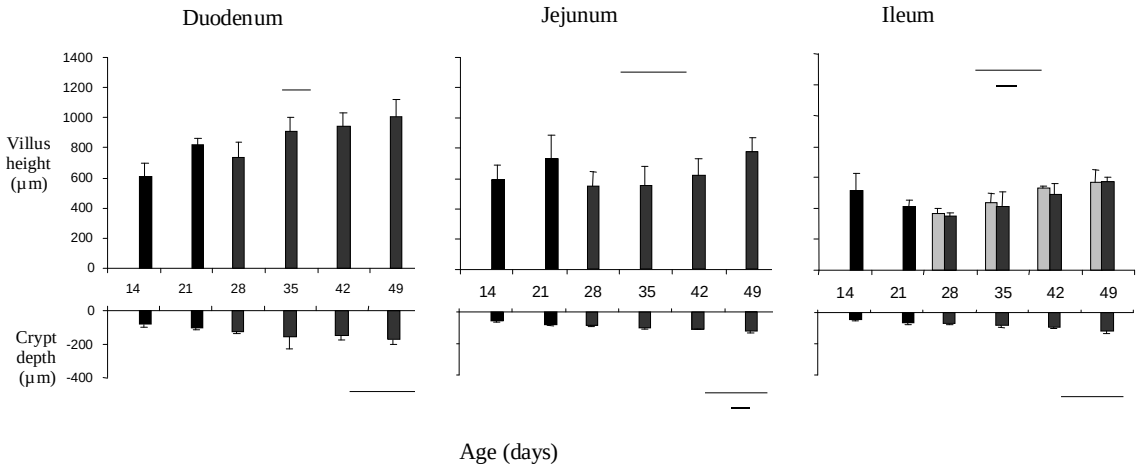


Figure 4. *Villus height and crypt depth along the small intestine in rabbits weaned at 35 days of age (from Gallois et al., 2005).*

and small intestine under the action of enzymes secreted by the rabbit (salivary glands, stomach, intestinal epithelium, and pancreas). Additionally, the digestive flora, especially those of cæcum and colon possesses numerous enzymatic activities able to hydrolyse the nutrients escaping the intestinal absorption. The development of the enzymatic system of rabbits depends mainly on ontogenic factors but it could be modulated by some extrinsic factors, as the age at weaning or feeding and nutrition (Debray *et al.*, 2002; Gutierrez *et al.*, 2002; Gallois *et al.*, 2005). On the opposite, the development of bacterial activity depends mainly on nutrients entering the cæcum and consequently on diet digestibility (Gidenne and Fortun-Lamothe, 2002).

The digestion in the small intestine results from pancreatic enzymes secreted in the intestinal lumen (amylase, lipase, trypsin, chymotrypsin) and from enzymes secreted by intestinal mucosa (maltase, saccharase, lactase…). Proteolytic activity in young rabbit's stomach is ensured by rennin at birth (Henschel *et al.*, 1972) and then, from 2 to 5 weeks after birth, by pepsin (Bernadac *et al.*, 1991; Dojana *et al.*, 1998). The gastric lipase activity is high from birth, as the lipids in the milk (10-25% on fresh basis) are the main source of energy. It reaches a maximum level in the 30 day old rabbit and then decreases between 30 and 60 days and is no more measurable in the adult (Bernadac *et al.*, 1991). The relative total intraluminal activity of amylase and maltase doubles between 25 days and 42 days of age (Scapinello *et al.* 1999; Debray *et al.* 2001). The development of the trypsic and chymotrypsic activity of the pancreas is linear between 25 and 52 days of age, and seems to depend mainly on ontogenetic factors (Debray *et al.*, 2002). The activity of lipase in intestine (of pancreatic origin) increases linearly from 25 to 52 days and could be modulated by diet (Debray *et al.*, 2002; Gallois *et al.*, 2004).

Degradation of the digesta entering the caecum by microorganisms results mainly in production of volatile fatty acids (VFA), ammonia, carbon dioxide, methane and hydrogen. Caecal fibrolytic activity is not detectable in young rabbits of 2 weeks of age. Cellulolytic activity progressively reaches its maximum level around 35 days of age and remains stable thereafter. On the opposite, xylanasic and pectinolyctic activity seems to increase between 10 and 24 weeks of age (Pinheiro *et al.*, 2001). Amylolytic flora is already active at 2 weeks of age and seems stable between 15 and 49 days of age (Padhila *et al.*, 1995). Consequently, caecal VFA concentration increases progressively (40 to 70 mmol/L see subchapter 4.2). Acetate levels (75-85%) are always higher than propionate (6-8%) or butyrate levels (6-10%). The fermentation pattern (e.g. VFA proportions) changes with age (Bellier *et al.*, 1995), showing an inversion of propionate/butyrate ratio, which becomes lower than 1 after 25-30 days of age (Padilha *et al.*, 1995; Zomborsky-Kovacs *et al.*, 2000; Gidenne *et al.*, 2002). Ammonia concentration in the caecum slightly falls with age (Gidenne and Fortun-Lamothe, 2002). Both increase in volatile fatty acids and decrease in ammonia concentration induce a fall in caecal pH from 15 to 42 days of age.

3.2. Maturation of the digestive immune system

Many factors ensure the defence of the gut mucosa against pathogens. Some factors do not belong to the immune system such as peristalsis, the permanent renewal of digestive epithelium, the mucus which contains several substances with a bacteriolytic or bacterostatic activity (lactoferrin, lactoperoxidase and lysozyme; Schroder, 1999) and

the competition between commensal and pathogens microflora (Berg, 1996; Kudsk, 2002). When these non-immunological mechanisms do not allow the elimination of the causative agent, the immune system is activated. Indeed, the digestive mucosa, as well as all others mucous membranes of the body, is associated with a lymphoid tissue. This tissue, called GALT (Gut Associated Lymphoid Tissue) ensures the defence of the host by neutralisation of pathogens but also the protection of mucosa by controlling the inflammatory response.

The overall organisation of the rabbit lymphoid system is similar to that of other mammalian species except for two additional structures, the *sacculus rotondus* located at the ileo-caecal junction, and the *vermiform appendix* located at the caudal end of the caecum, which have been identified only in this species (Mage, 1998). The natural (or innate) primary immune response, which is non-specific, represents the first line of defence against pathogens and takes place all along the digestive tract. On the opposite, the adaptive (or acquired) immune response, which is directed against a specific foreign element in the gut, is played by the induction sites (identification of agents and activation of cells starting the reaction against antigens) and the effector sites (elimination of undesirable agents; Drouet-Viard and Fortun-Lamothe, 2002). The induction sites contain a lot of lymphoid cells organised in lymphoid follicles, such as Peyers'patches. In the rabbit, there are between two to ten Peyer's patches along the small intestine (Mage, 1998). They are composed of numerous dome-follicles which extend into the lumen of the gut (see Figure 3.). The dome-follicles contain primarily B cells producing IgM, and also macrophages and CD4-T cells (Ermak *et al.*, 1994).

The interfollicular regions, between dome and germinal centre, are T-cell rich areas (Hein, 1999). The dome is covered by a specific epithelium, the FAE (Follicle Associated Epithelium) containing specific M cells, and is designed for the uptake of macromolecules, particles and micro-organisms by transepithelial transport (Neutra, 1999). Both *vermiform appendix* and *sacculus rotondus* contain several hundred dome-follicles and their organisation is quite similar to that of Peyer's patches (Mage, 1998).

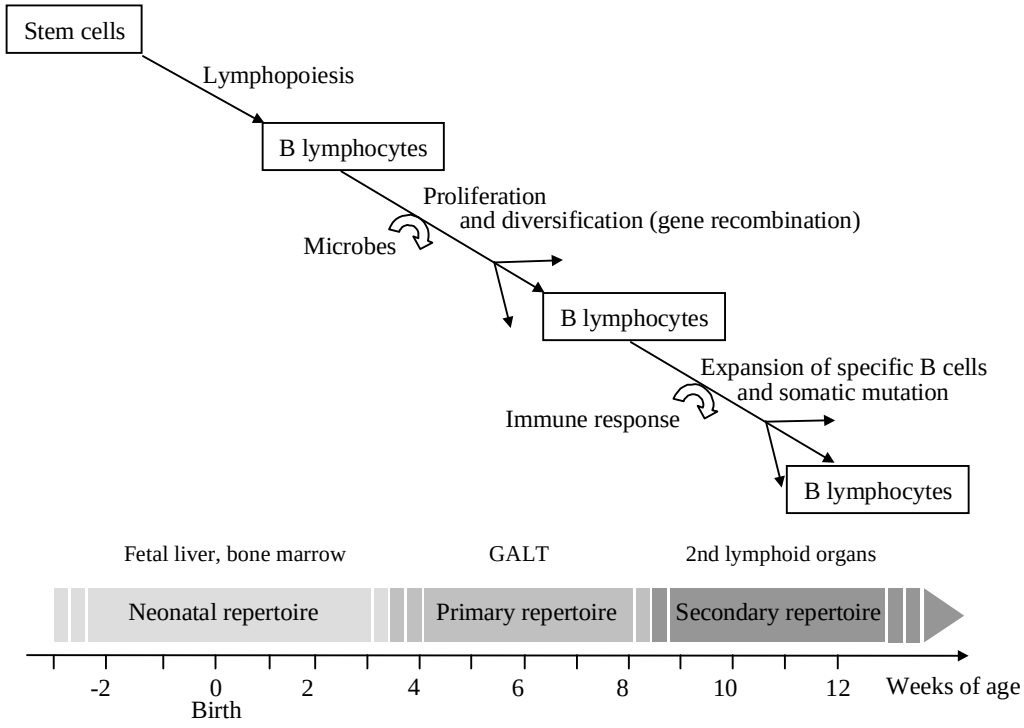


Figure 5. *Antibody repertoire development in rabbit.*

Peyer's patches, the *vermiform appendix* and the *sacculus rotundus* are specialised in the uptake of macromolecules and microorganisms from the gut lumen to the lymphoid cells (Simecka, 1998). M cells of the follicle associated epithelia capture luminal antigens by macro-pinocytose and transfer them intact to underlying dendritic and macrophage populations. They present the processed antigen to CD4+ T cells while naive B cells recognise directly the intact antigen. After the B and T cells are sensitised to the antigen, they move towards the mesenteric lymph nodes where they mature and

proliferate. Activated cells thus migrate through the thoracic duct into the circulation and home to the epithelium (intraepithelial lymphocytes) or to the *lamina propria* (lamina propria lymphocytes) of the digestive tract and form the effector tissue. The effector cells diffuse along the intestine tract allowing the development of the immune response throughout the digestive tract. The *lamina propria* B cells are transformed into plasma cells secreting IgA, which is the predominant type of immunoglobulin secreted in the gut (Lamm, 1997). Unlike other immunoglobulins classes, they do not activate complement or inflammatory responses, which make them ideal for protecting mucosal surfaces. They realise immune exclusion by cross-linking microorganisms or macromolecules thus preventing their contact with the surface of epithelial cells (Corthesy and Kraehenbuhl, 1999).

The immune system can identify a great number of antigens and produces a wide antibody repertoire either secreted by B cells or as receptors of T cells. Three mechanisms allow the diversification of antibody repertoire: the multiplicity of genes coding for variable regions, somatic recombinations and somatic mutations (Roitt and Delves, 1998). Each species uses these different strategies in a more or less important way. Rabbits have the particularity of generating their antibody repertoire in three stages (Knight and Crane, 1994; Figure 5).

First, a **neonatal antibody repertoire** is established by B cells generated during B lymphopoiesis early in rabbit life (before 3 weeks of life). The **primary antibody repertoire** develops between 4 and 8 weeks of age in the gut-associated lymphoid tissue. Several experimental data suggested that intestinal microbial flora is required during development of the primary antibody repertoire. (Lanning *et al.*, 2000a, b; Vajdy *et al.*, 1998). The primary repertoire, which provides the rabbit a diverse collection of antibody specificities, represents the unique reservoir of B cells for the whole life of the animal. This repertoire is then modified in adult life during antigen-specific immune responses to give the secondary repertoire. Therefore, in rabbit, the maturation of digestive immune system continues until 8 weeks of age under the influence of digestive flora.

3.3. Methods to estimate the digestive capacity in the young rabbit.

Methodology to estimate the digestive capacity in the rabbit has been developed essentially to estimate the faecal digestibility of the dry matter in the growing rabbit (6-10 weeks of age), and has been standardised by the EGRAN group (Perez *et al.*, 1995, 1996). These methods are precise enough, since the intake and the faecal output are relatively steady, after 6 weeks old. However, estimating the digestive capacity of 3-5 weeks old rabbits is more questionable, since the intake is increasing rapidly. It is also associated with an increase of the digestive contents that could lead to underestimation of the faecal output (Parigi Bini *et al.*, 1991). For instance, when using the European reference method (Perez *et al.*,1995), the digestibility coefficient measured before weaning is frequently higher than the one measured at 6 weeks old (Debray *et al.*, 2000). This is not compatible with the digestive maturation with age. Therefore, new procedures for measuring digestion in the young should be developed, and particularly in the case of an early weaning. These methods should be based on corrections to simulate steady-state conditions (Parigi Bini *et al.*, 1991; Gidenne *et al.*, 2005), or should use markers to evaluate more precisely the real balance between intake and faecal output (Gallois *et al.*, 2006).

4. Impact of breeding technique on digestive maturation

Breeding techniques that modify the intake pattern, such as age at weaning or feed restriction, are also able to modulate the digestive function.

4.1. Age at weaning and role of milk/solid feed intake

As reported above (under 2.2), solid feed intake before weaning is inversely related to milk intake, and thus the digestive maturation is also modified. For instance, feeding young rabbits exclusively with milk, beyond the classical weaning age (till 42d), sharply inhibits the implantation of the fibrolytic flora, and the fermentative profile is specific of a proteolytic metabolic activity (with high NH_3 level), associated with a very low caecal VFA concentration and a high pH (Padilha *et al.*, 1999). Similarly, Zomborszky-Kovács *et al.* (2000) reported a slower development of the caecal microbial activity in double-suckled kits compared to single-suckled ones. Gyarmati *et al.* (2000a,b) also reported a higher digestive development (length and volume of organs) for double-suckled young, that was mainly correlated to the level of dry feed intake.

Reversely, an early removal of milk (from 18-25 days of age) in an early weaning or a lower milk intake stimulated the solid feed intake and, is followed by a higher concentration of VFA and a lower pH in the caecum, compared with animals of the same age which are receiving only milk (Maertens and Piattoni, 2001; Xiccato *et al.*, 2003). However, milk/solid feed intake weakly affects the morphology of the small intestine mucosa (Gallois *et al.*, 2005), or the amylolytic and maltasic activity and the digestive efficiency after weaning (Scapinello *et al.*, 1999).

4.2. Feed intake level and digestive maturation

The role of feed intake level was mainly studied by comparing *ad libitum* vs restricted feeding. The effect of reducing the feed intake level concerns mainly the growth and tissues development (muscle, bone, fat). During the restriction, the total digestive efficiency was not affected (Xiccato *et al.*, 1992; Diaz Arca *et al.*, 1999). In return, 5 hours after meal distribution, the caecal VFA level increased linearly with the feed restriction level (from 100 to 60%, Gidenne *et al.*, 2004), and this may explain the favourable effect on digestive health observed for restricted rabbits (see 4.3.3). Reversely, when the intake is decreased through an excess of dietary methionine (without restriction technique) Gidenne *et al.* (2002) reported a lower fermentative activity in the young rabbit, but without change in the fibrolytic potential of the flora. Finally, the effects of feed intake level on the digestive maturation need to be deeply explored, particularly to identify the physiological mechanisms explaining their favourable effects on digestive health.

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4.2. The digestive ecosystem and its control through nutritional or feeding strategies

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1. Introduction

The colonization of gastrointestinal microbiota is essential to maintain intestinal health and rabbit nutrition by preventing colonisation of pathogenic bacteria, modulating the immune system and degrading non absorbed substrates in the small intestine producing nutrients for the animals. However, this colonisation may be also the cause of different digestive pathologies. The main enteric pathologies in rabbits are of multi factorial origin with many factors acting in cascade (Peeters *et al.*, 2000). Changes in the housing conditions (type of cage, temperature, etc) and in the nutrition together with an incomplete development of the defensive mechanisms in the animal, might favour the outbreak of the most frequent dysbiosis (colibacillosis and clostridiosis) described in the rabbit around the weaning.

There is a general agreement that the nutrition can affect the incidence of pathological processes of enteral origin. The supply of unbalanced diets has been related with the appearance of digestive disorders by means of two mechanisms: i) promoting a higher retention time of the digesta in the digestive tract or ii) causing a higher flow of easily available substrates into fermentative area. In both situations, the alteration of the intestinal microbiota has been postulated as the possible primary cause of these pathologies. In the case of the rabbit, the factors of the diet more related with the appearance of diarrhoeas are the level of starch and fibre, usually inversely correlated, the type of fibre and the level and type of protein (De Blas *et al.*, 1981, Blas and Gidenne, 1998: de Blas *et al.*, 1999; Gidenne, 2003). However, the relationship between these nutrients and the intestinal microbiota (pathogenic or commensal bacteria) has not yet been well established.

Understanding the role of the nutrition on digestive health needs a correct characterisation of the mechanisms implicated in the competitive exclusion of pathogens by the commensal bacteria and the mechanism of pathogenicity. Traditionally the nutritional experiments have tried to characterise these mechanisms by measuring the end products of the microbial activity (VFA etc...) or the physico-chemical parameters of the ecosystem (pH) or the enzymatic activity of intestinal microbiota. However, few studies have been devoted studying the effect of nutrition on the colonisation of both commensal and pathogenic bacteria. The use of classical techniques of bacteria cultivation implies a large number of assays to characterise the microbiota and lead to a largely incomplete flora pattern. The new molecular techniques can be useful to understand the complexity of this relationship.

The aim of this chapter is to review the information about the relationship between nutrition and the microbiota and the use of different strategies for microbial characterisation.

2. Colonisation of intestinal tract

In all mammals intestinal microbiota is poorly understood. This is due to the fact that the intestinal flora is composed of a huge number of bacteria with a great biodiversity: for example the digestive tract in the man, harbours approximately 1014 bacteria belonging to more than 400 different species, almost all being strictly anaerobes (99%) ; in addition, 70-

80% of this microbial biomass is not-cultivable (Suau *et al.*, 1999).

In rabbit, the knowledge on colonisation of the gut by the microbiota, acquired with cultured-based techniques, concerns old works. Nevertheless, more recent data on bacterial species not yet described in this animal have been obtained, in particular thanks to molecular biology-based techniques.

The microbiota evolves according to the age and the intestinal segment. In the young rabbit, the implantation of the flora varies along the intestinal tract and its composition evolves with age (Smith, 1965; Gouet and Fonty, 1973; 1979) and time after weaning (Padilha *et al.*, 1995). The originality of this microflora is that it is established slowly and that it presents a simple composition dominated by strictly non-sporulated anaerobic bacteria.

2.1. Implantation

One of the characteristics of the microbiota is the very slow and irregular appearance of strictly and facultative anaerobic bacteria in the digestive tract, associated with important individual variations, especially in the high parts of the intestine (Gouet and Fonty, 1979). During the first days that follow the birth the digestive tract of the animals is almost sterile and it is only starting from the end of the first week of life that an abundant population is observed at the level of the cæcum. These results join those of Smith (1965) who could not isolate bacteria from young rabbits of less than 4 days, whereas in the majority of the other animals, like rat, chicken or piglet, an abundant microbiota settles in the digestive tract following ingestion of the first meals (Smith, 1965; Sinkovics and Juhasz, 1974).

2.2. Colonisation of the stomach

Gouet and Fonty (1979) showed that 75% of the young rabbits do not harbour any flora in the stomach during the first 15 days of life, in spite of the existence of a slightly acid pH (4.5 to 5).

This phenomenon would probably be related to an antibacterial factor present in the milk of the doe and identified as being octanoic and decanoic acids (Canas-Rodrigues and Smith, 1966; Cole *et al.*, 1983). This "antibacterial" factor in the stomach could thus delay the establishment of the flora which becomes constant only when the young rabbits start to consume solid food, around day 17 of age. At this time, the process of caecotrophy begins and the bacteria present in the stomach would be the result of the recycling of the flora coming from the soft faeces. The rabbits, whose caecotrophy is prevented, have sterile stomach contents (Smith, 1965). In spite of the daily re-ingestion of several billion of bacterial cells due to caecotrophy, the level of the flora in the stomach remains low after weaning (10^4 to 10^6 bacteria/g of contents), compared with that found in the majority of the rodents (10^8 to 10^9 bacteria/g of contents) (Raibaud *et al.*, 1966; Ducluzeau, 1969). It originates probably from the very acidic environment of the stomach content (pH<2.0, see 4.1)

2.3. Colonisation of the small intestine

According to Gouet and Fonty (1979), the colonisation of the small intestine is faster than that of the stomach. The microbiota is present as soon as the first week of life and regularly increases until the 7th day after the birth, with values 10 to 100 times higher than in the stomach with notable individual variations. The proportion of facultative anaerobic bacteria is sometimes a little higher before weaning, and after weaning this same flora is generally absent.

2.4. Colonisation of the cæcum and the colon

In 2 or 3-day-old animals, the total number of bacteria present in the cæcum varies considerably according to individuals. At the end of the first week of life, the cæcum harbours an abundant flora (10^7 to 10^9 bacteria/g, using cultivation methods) which increases from the 2nd week (10^9-10^{10} bacteria/g) and of which the amplitude of the individual variations becomes weaker. During this period, the number of facultative anaerobic bacteria is sometimes equivalent to that of the strictly anaerobes. From the third week, the number of facultative anaerobic bacteria falls dawn to 10^2-10^4 and it is not rare that this microbiota is absent after weaning (Ducluzeau, 1969), whereas the strictly anaerobic flora remains stable to 10^9-10^{11} bacteria/g (Zomborszky-Kovacs *et al.*, 2000). In the colon, the microbiota follows an evolution identical to that of the cæcum but the total number of bacteria there is systematically lower (Gouet and Fonty, 1979).

In addition, Emaldi *et al.* (1979) have shown that the total microbiota of the cacao contents and that of the soft faeces are similar (approximately 10^{11} bacteria/g), whereas in hard faeces, the number of bacteria is 10 times weaker. Bonnafous and Raynaud (1968) indicate that the number of bacteria present in faeces do not exceed more than 30% of what is present in cæcal contents and suggest that this phenomenon could be related to the existence of a "lytic factor" secreted by the colon.

2.5. Microbiota composition

Using molecular techniques, we recently found that the first characteristic of healthy rabbit caecal microbiota is an absence of protozoa and weak prevalence of fungi, contrary to the ruminants (Bennegadi *et al.*, 2003). Some fungi, such as yeast (*Saccharomycopsis guttulatus*) would be found in the caecum of healthy rabbit (Peeters, 1988). However, in diseased animals, ciliate protozoa may be found in the caecal content (Lelkes *et al.*, 1987). Eimeria spp, other intestinal protozoa, are generally present in rabbit for meat production (Lebas *et al.*, 1986).

2.5.1. Facultative anaerobic microbiota

The second characteristic of the digestive microbiota is that the facultative anaerobic microbiota in the young rabbit has a simple composition dominated by Streptococcus until the 14th day of life, whereas enterobacteriacea are detected only occasionally (Gouet and Fonty, 1979). Lactobacilli are generally absent (Cole *et al.* 1983; Gouet and Fonty, 1973; 1979; Penney et al,. 1986).

As soon as solid food is consumed, the flora changes with a reduction of the number of Streptococci and the appearance of enterobacteria. But the diet is not the sole factor that plays a role on the microbiota. Indeed, other factors can be involved. The capacities for absorption of the intestine evolve with the age: many enzymes become functional (Henschel, 1973; Lebas et a1., 1971) and will act on food. The changes of the anaerobic flora themselves probably play a role which still remains difficult to be defined at the moment.

After weaning, facultative anaerobic bacteria isolated from the intestinal tract belong mainly to the Gram-positive genera Bacillus, Enterococcus and Staphylococcus and Gram-negative Enterobacter or *Escherichia coli* (Forsythe and Parker, 1985; Canganella *et al.*, 1992). Regarding *E. Coli*, in fact two populations of rabbits can be considered: those (about 30% of the rabbits) without any detectable *E. Coli* ($< 10^2$ bacteria/g) and those with a coli flora of 10^4-10^5 bacteria/g (Padilha *et al.*, 1996). Lactobacilli are exceptionally found (Yu and Tsen, 1993).

2.5.2. Strictly non-sporulated anaerobes

The third characteristic of the microbiota is the very clear prevalence of the strictly non-sporulated anaerobic Gram-positive bacteria, Bacteroides, along the digestive tract (Gouet and Fonty, 1973; 1979; Penney *et al.*, 1986), while anaerobic sporulated bacteria belonging to the genera Clostridium, Endosporus and Acuformis are 100 to 1000 times lower (Gouet and Fonty, 1979). These strictly anaerobic bacteria (analogous to an equivalent number of Streptococci) are established quickly in the intestine of the young rabbit. In the majority of the cases, they constitute the dominant flora as soon as the 2nd week of life, whereas in the majority of other mammals they appear only at weaning (Lee *et al.*, 1971; Raibaud *et al.*, 1966). It seems that the genera Endosporus before weaning, and Acuformis later, constitute with Bacteroides the dominant flora in the cæcum and colon of rabbit. Contrary to Trovatelli *et al.* (1974) and Ducluzeau *et al.* (1975), Gouet and Fonty (1979) never found Bifidobacterium.

By using dot-blot hybridization with 16S rRNA targeted oligonucleotides probes, Bennegadi *et al.* (2003) state that bacteria and archaea represent respectively 73% and 22% of the total microbial communities in the caecum at weaning. They also demonstrated the predominance of the Flexibacter-Cytophaga-Bacteroides group and the presence of four cellulolytic species, usually identified in ruminants: *Fibrobacter succinogenes*, *F. intestinalis*, *Ruminococcus albus* and *R. flavefasciens*, this last species being dominant.

3. Microbial activities

Emaldi *et al.* (1979) are among the first authors to be interested not only in the composition of the flora but also in its metabolic activity i.e. in its capacity to degrade the substrate entering the caecum. Thus, microbial activities have been classified according to their decreasing importance: ammonia use, ureolytic, proteolytic and cellulolytic. Crociani *et al.*, 1984 and Forsythe and Parker (1985) confirm that the caecum is the site of an important ureolytic activity due to ureolytic aerobes and anaerobes. Amylolytic flora is present as soon as day15 of life, i.e. before the rabbit consumes starch, at a high level 10^{10}-10^{11} bacteria/g and then, does not decrease (Padilha *et al.*, 1995).

Fibrolytic flora (hydrolysing plant cell-wall polysaccharides such as cellulose, xylanes and pectines) appears around 14 days after birth and reached 6.103 before weaning (Boulharouf *et al.*, 1986, Padilha *et al.*, 1995), whereas in sheep for example it is present from birth (Fonty *et al.*, 1987). In adult rabbit the cellulolytic flora would vary from 10^4 to 10^7 bacteria/g of cæcal contents (Boulharouf *et al.*, 1991; Padilha *et al.*, 1995). It can be noted that the cellulolytic flora does not develop in rabbits exclusively fed with milk, up to 42 days of age (Padilha *et al.*, 1999). The strains implied in the cellulolytic activity are mainly Eubacterium cellulosolvens while those concerned by xylanolytic and pectinolytic activities are assigned to the species Bacteroides ruminicola (Boulharouf *et al.*, 1991).

4. Methodological approaches for analysing the digestive microbial flora

There are up to 10^{14} total bacteria in the intestinal tract of animals. The composition and the activity of this microbiota have a strong influence on health and disease through their participation in the nutrition, pathogenesis, and immune function of the host (Gibson and Roberfroid, 1995). The gut microbiota, or the overall microbial species that are present in the intestinal tract of animals, is composed of more than 40 genera and some hundred species (Mackie *et al.*, 1999). Only twenty-five to forty per cent of the microbial species present in this complex microbiota can be cultured *in vitro* (Langendijk *et al.*, 1995; Suau *et al.*, 1999; Tannock *et al.*, 2000), a characteristic that makes its knowledge by the researchers difficult, and consequently the possibility of its control by feed composition.

Although some of the non-cultivable microorganisms may be non-viable, it is likely that

many are viable but non-cultivable due to their fastidious requirements for anaerobiosis or, more likely, due to the complex nutritional interactions that can occur between the components of the intestinal microbiota.

Table 1. Selective culture media for different bacterial species and rate of normal faecal plate count.

Selective Growth Media	Plate Count (CFU/g)	Target Group
Nutrient agar	10^2-10^8	Total aerobes
MacConkey agar	10^2-10^7	Coliforms
Wilken-Chalgren agar	10^5-10^{10}	Total anaerobes
Bacteroides agar	10^5-10^{10}	*Bacteroides* spp.
Beeren's agar	10^5-10^{10}	*Bifidobacterium* spp.
Azide/Crystal violet agar	10^2-10^7	*Enterococcus* spp.
Fusobacterium agar	10^3-10^8	*Fusobacterium* spp.
Raffinose-Bifidobacterium agar	10^5-10^{10}	*Bifidobacterium* spp.
Clostridia agar	10^4-10^9	*Clostridium* spp.

(adapted from Rastall and Gibson, 2002)

As can be deduced from Table 1, only some bacterial species can be examined by classical microbiological methods, since no more than 1/100 to 1/10 of the total intestinal bacteria are cultivable. Therefore, molecular microbiology techniques are presently extensively used to provide a more extensive view of the digestive flora in animal digestive ecosystems, including parameters for biodiversity and stability. For instance, using non-specific probes to the 16S rRNA genes and FISH method (see below), the number of bacteria in human faecal samples is approximately ten-fold higher than estimated using standard culture techniques (Harmsen *et al.*, 2000; Langendijk *et al.*, 1995). It is also possible to identify different gut microorganisms by amplification of the gene encoding 16S-rRNA and analysis by restriction fragment length polymorphism or by sequence of the amplified fragment (Collins and Gibson, 1999). As we can see later, the majority of the molecular techniques used for the analysis of gut microbiota are based on 16S-rRNA gene analysis.

The most useful molecular techniques are:

1. Restriction fragment length polymorphism (RFLP)
2. Denaturant gradient gel electrophoresis (DGGE), or Single Strand Conformation Polymorphism (SSCP).
3. Ribotyping.
4. Ribosomal DNA sequencing.
5. Direct amplification.
6. Genetic probes.
7. Flow cytometry.

4.1. Restriction fragment length polymorphism or RFLP

The 16S-rRNA gene is located in the chromosome of all the prokaryotic cells, and in the genetic material present in mitochondria or chloroplast of eukaryotic cells. Only some anaerobic protozoa don't have any 16S rDNA, but to analyse these microorganisms the amplification of 30S rRNA can be used.

The 16S rRNA molecule has two additional characteristics, related with its primary structure, that confer its potentiality in the study of complex microbiota, as gut microbiota has some highly conserved areas at the same time as some hyper-variable areas. The first characteristic allows the use of some universal primers that lead to the amplification of a high percentage of microorganisms. The second characteristic enables the resolution between microorganisms, especially by changes in the recognition sequences of restriction nucleases. The combination of DNA amplification of 16S rDNA, digestion with restriction nucleases of amplified DNA, and analysis by agarose gel electrophoresis becomes an excellent system to study the composition of gut microbial components without the necessity of *in vitro* cultures (Liu *et al.*, 1997; Suau *et al.*, 1999).

By this technique, the total DNA extracted from gut samples is amplified by PCR using universal primers – primers that amplify a great proportion of the intestinal microbial component – some of them published by Lane (1991). The DNA fragments amplified by PCR are digested with restriction endonucleases, generally with the also called tetrameric restriction enzymes as *Alu* I, *Rsa* I or *Hpa* II. Finally, the restriction fragments were resolved using agarose electrophoresis. Each bacterial genus, and in some cases bacterial species, is characterised by a particular pattern of electrophoretic bands.

The recognition capacity of RFLP technique is based on the comparison of the electrophoretic profile obtained with real samples (Figure 1) with data bases constructed with the theoretical segments obtained with the same restriction enzymes on

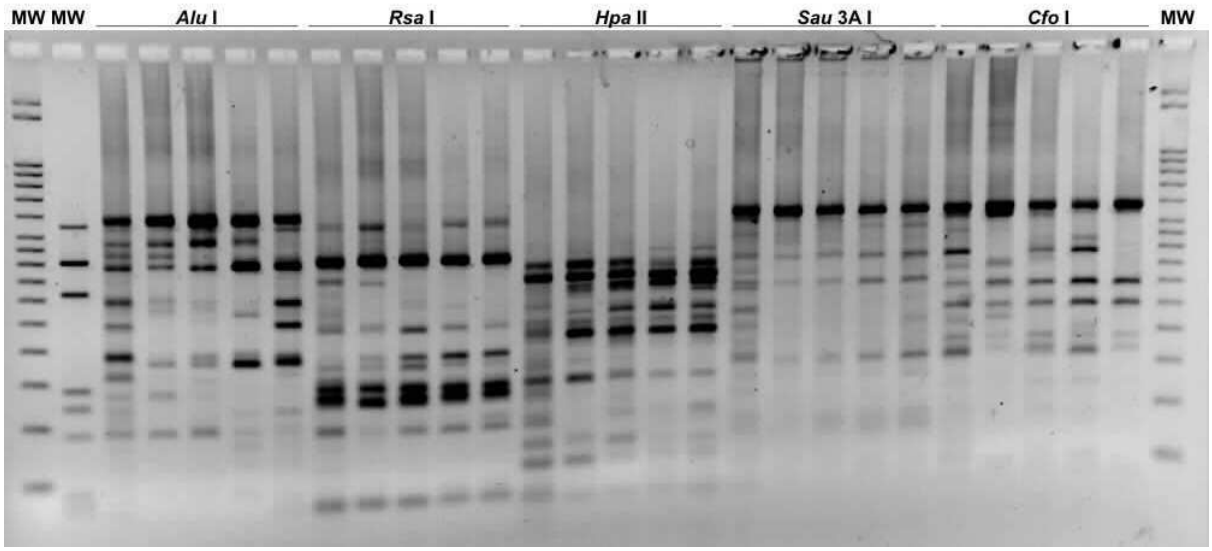


Figure 1. *Electrophoretic profiles for DNA of caecal samples of rabbits amplified with universal primers for 16S rRNA genes and digested with Alu I, Rsa I, Hpa II, Sau 3A I or Cfo I restriction enzymes.*

sequences of 16S r-RNA genes deposited in the Ribosomal Database Project (Maidak *et al.*, 2001).

With RFLP methodology is possible to recognise the specific profile of some pathological conditions by comparison of the profile of samples from normal rabbits and samples from pathological condition together the profile of the cultured pathogen (Figure 2). In this figure the RFLP bands related with *Escherichia coli* increment their intensity in colibacillosis when compared with normal rabbits.

4.2. Denaturant gradient gel electrophoresis or DGGE

This technique allows to assess the bacterial genetic diversity, using the analysis of the V3 region of 16S rDNA PCR products (approximately 200-700 bp) obtained with primers specific for the domain Bacteria (Simpson *et al.*, 1999).

The separation principle of DGGE is based upon the melting (denaturation) properties of DNA in solution. Initially the fragments move according to molecular weight, but as they progress into higher denaturing conditions, each (depending on its sequence composition) reaches a point where the DNA begins to melt. The partial melting severely retards the progress of the molecule in the gel and a mobility shift is observed. DNA molecules melt in discrete segments called melting domains, when the temperature or denaturant concentration is raised. The melting temperature (Tm) of a double stranded DNA fragment is influenced by hydrogen bonds formed between complementary base pairs, in other words the ratio of GC to AT base pairs, and also by the attraction between neighbouring bases on the same strand (known as stacking interactions).

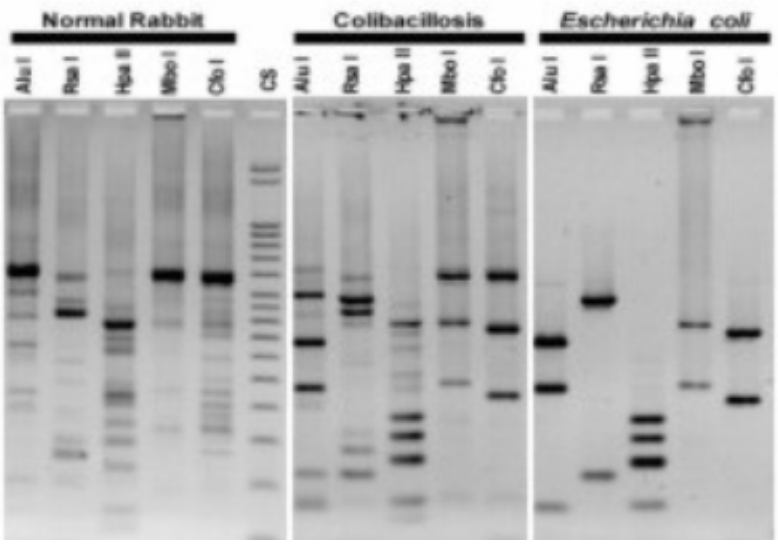


Figure 2. *RFLP profile from intestinal samples of normal rabbits, rabbits with colibacillosis and the Escherichia coli strain isolate from the intestinal colibacillosis.*

As the DNA sample progresses through the gel, from a low denaturant concentration to a higher one, it starts to melt at varying points. The higher the GC content of the sample, the harder it is to melt. Thus the DNA sample is able to progress further into the gel before stopping. Samples with lower GC content melt more rapidly. Therefore, they progress slower within the gel, thus becoming separated from the other faster moving strands of DNA.

Complete denaturation is prevented by the presence of a high

melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is produced by using a primer with a 5' tail consisting of a GC-rich sequence of around 30-50 base pairs in the PCR (Myers *et al.*, 1985).

Profiles generated by RFLP or by DGGE can be analysed by comparing the presence or absence of individual bands as well as measuring the intensity of a band within a profile, where the intensity can be related to the relative abundance of a sequence within a sample, although at best this is considered a semi-quantitative measure.

4.3. Ribotyping

A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome which contain rRNA genes. To obtain a ribotype of a micro-organism, it must first be cultured to obtain enough cells for the procedure.

Total DNA is isolated and digested into multiple fragments, of sizes between 1 kb to 20 kb, using restriction enzymes with a frequently occurring recognition sequence, generally a 6 bp recognising enzymes that have a random recognition frequency of 1:4096 bp. The restricted fragments are separated by agarose gel electrophoresis and subsequently hybridised with a probe targeted to either the 16S, 23S or 5S rRNA genes. In practice, probes to the 16S rRNA are the most commonly used. The hybridisation can be carried out directly in the gel using in gel hybridisation techniques, or alternatively on a nylon or nitrocellulose membrane following Southern transfer of the DNA from the gel to the filter.

The basis of the technique is that bacteria generally contain multiple copies (up to eight or more) of the rRNA genes throughout their genome, thus enabling the RFLP to be obtained. However, some bacteria contain as few as one copy of rRNA genes, thus limiting the effectiveness of ribotyping for fingerprinting these bacteria. Bacteria with a single copy of the rRNA operon are usually slowly growing bacteria. Restriction bands containing copies of the rRNA genes are visualised and the pattern of the band sizes represents a characteristic fingerprint of each bacterial species.

An advantage of ribotyping is that a single rRNA probe can be used to type all bacteria. It is also very reproducible and its effectiveness for the analysis of human intestinal microbiota has been demonstrated (Mangin *et al.*, 1995; McCartney *et al.*, 1996). This technique has the limitation in that it requires the culturing of bacteria and is labour intensive.

4.3.1. Ribosomal DNA sequencing

Accurate typing of unknown isolates is possible to achieve by sequence analysis of the gene of 16S ribosomal RNA (rRNA). This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese (1987). The available database of rRNA sequences is now extensive, which allows detailed studies to be made on the phylogenetic position of unknown isolates. This molecular phylogeny approach has revolutionised the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned (Olsen *et al.*, 1994). Technically, this is well feasible as the polymerase chain reaction (PCR) can be used to directly amplify the 16S rRNA gene directly from colonies using primers that are directed at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is ~ 1.5 kb can then be directly sequenced and compared to the rRNA database (Maidak *et al.*, 2001).

Few studies on intestinal microbiota of rabbits have been conducted by analysis of the sequences obtained by PCR amplification, using universal primers of 16S rRNA gene, of the total DNA extracted from caecal contents of normal rabbits. The amplified segments were cloned in the appropriate system and different clones were randomly selected and sequenced to obtain an image of the most frequent microorganisms present in the rabbit intestine (Pérez de Rozas *et al.*, 2004; Abecia *et al.*, 2005). With these studies we can infer that, as expected, the majority (66%) of the components of rabbit microbiota is unknown/ uncultured (Table 2).

Table 2 Identity of 50 random fragments obtained by amplification with universal primers for 16S rRNA genes of total DNA extracted from caecal samples of rabbits.

Bacterial species	Number of clones	Percentage
Bacteroides fragilis	9	18%
Bacteroides spp.	6	12%
Enterococcus ratti	2	4%
Uncultured bacterium, clone HuCB3	5	10%
Uncultured bacterium, clone HuCA18	5	10%
Uncultured bacterium cadhufec17c09sav	3	6%
Uncultured bacterium adhufec250	2	4%
Uncultured bacterium	18	36%

4.3.2. Direct amplification

In recent years different teams working on digestive physiology, pathology or on the development of new probiotics, have developed multiple pairs of primers to analyse the presence or absence of different bacterial species, and in some cases particular strains of species of interest. With these

primers, PCR methodology and electrophoresis in agarose of the amplified fragments is possible to analyse symbiotic or pathogen microorganisms presents in intestinal samples. Some tandems of primers that specifically amplify some intestinal microorganism are listed in the Table 3.

Table 3. Primers used to recognise different bacterial species, or specific bacterial virulence genes, found in the gut of rabbits, and size of the amplified fragments.

Primers	Target gene/microorganism	PCR product size (bp)
5'-GTGGCGAATACTGGCGAGACT-3' 5'-CCCCATTCTTTTTCACCGTCG-3'	*eae* gene of *Escherichia coli*	891
5'-ACCCACGCCCAACATAGAC-3' 5'-CCACGAGCAGGAAGAAAGG-3'	*Prevotella-Bacteroides*	424
5'-CACGTATCCAACCTGCCCTT-3' 5'-AGCGGTGATTGCTCACTGAC-3'	*Bacteroides fragilis*	915
5'-GGCAGCATTTCAGTTTGCTTG-3' 5'-GGTACATACAAAATTCCACACGT-3'	*Bacteroides thetaiotaomicron*	423
5'-AAGATTTGTAAGGCGCTT-3' 5'-ATTTCCTGAAATCCACTC-3'	Alpha-toxin *Clostridium perfringens*	1167
5'-CCGCATGGCAGTGTGTGAAA-3' 5'-CTGCTGATAGAGCTTTACATA-3'	*Clostridium clostridiiforme*	255
5'-TGAGGAGACTGCCAGGGA-3' 5'-CTCCTTCTTTGCAGTTAGGT-3'	*Ruminococcus obeum*	312
5'-AACTCCGGTGGTATCAGATG-3' 5'-GGGGCTTCTGAGTCAGGTA-3'	*Peptostreptococcus productus*	268
5'-AACGATGAAGCTTCTAGCTTGCTAG-3' 5'-GTGCTTATTCGTTAGATACCGTCAT-3'	*Helicobacter* spp	400
5'-AGATGGCCTCGCGTCCGA-3' 5'-CCGAAGACCTTCTTCCTCC-3'	*Fusobacterium prausnitzii*	199

Recently, PCR has evolved to real-time PCR, a method that permits the quantification of the initial amount of the genetic material in the required samples. In general there is a linear relationship between the genetic material quantified by real-time PCR and the bacterial count, with higher results in real-time PCR, because some unviable bacteria have amplified DNA.

The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Lee, 1993; Livak, 1995). This signal increases in direct proportion to the amount of PCR product in the original reaction mixture. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

4.3.3. Genetic Probes

A genetic probe is a labelled single-stranded nucleic acid that can specifically hybridise (bind) to its complementary sequence. The target sequences are generally chosen so that they are unique to the particular genus, species or strain.

Technically, the procedure is rapid and easy because colonies can be directly probed, by lysing the colony to expose the nucleic acid content and allowing access for the probe. The label on the probe can be either enzymatic or fluorescent, which can be readily detected. The selection of the correct probes is the key to success with this method, because any cross reactivity can give ambiguous results. Probes can be obtained using either a shotgun or a directed approach.

The shotgun approach is to randomly isolate DNA fragments and test them for probe reactivity against a bank of isolated strains. This approach has been used to obtain strain- and species-specific probes for bifidobacteria (Mangin *et al.*, 1995) and a species specific-probe for *Bacteroides vulgatus* (Kuritza and Salyers, 1985).

The directed approach to probe selection leaves less to chance, as it relies on choosing probes directed at target sequences which are thought to be unique to the particular microbe or group of microbes under study. One strategy is to identify enzymes unique to a group of organisms and direct probes at targets within the enzyme gene sequence. A potential example would be the bifidobacterial enzyme, fructose-6-phosphate phosphoketolase, which is used by members of this genus to

metabolise carbohydrates via a unique pathway often called the fructose-6-phosphate shunt.

Generating short (20-30 bases) oligonucleotide probes directed against regions of the rRNA is the most common means to obtain genus- and species-specific probes. During the probe design process, probes can be tested against the extensive database of rRNA sequences using computer models. With the correct design procedure, the resulting probes should have a low cross reactivity.

Using this strategy, genus specific probes have been designed and evaluated for detection of *Bacteroides* (Doré *et al.*, 1998), *Bifidobacterium* (Kaufmann *et al.*, 1997), or *Clostridium* (Sghir *et al.*, 2000) from faecal samples. Species specific probes for bifidobacteria (Yamamoto *et al.*, 1992) or *Bacteroides* (Kreader, 1995) have also been developed.

The combination of genetic probes with the microarray technology will result in a powerful technique to analyse the variation of microbial population in the gut of animals under different conditions (age, feed components, feed additives, pathological conditions…)

A variation of molecular probes used in the analysis of the intestinal microbiota components is to hybridise fluorescently labelled oligonucleotide probes directly to cells fixed on a glass slide, known as fluorescent in situ hybridisation or FISH (Amann, 1995). The fixing process of samples permeates the cells to allow the short probes to access the nucleic acid inside the cell. This hybridization can be carried out on glass slides and the cells with the hybridised fluorescent probe can subsequently be visualised by fluorescent microscopy. Genetic probes targeting the major components of the gut microbiota are 5' labelled with Cy3, a fluorescent dye.

The most commonly used probes are Bact338, generic for the domain bacteria (Amann *et al.*, 1990), Bif164, specific for bifidobacteria (Langendijk *et al.*, 1995), Bac3003, specific for bacteroides (Manz *et al.*, 1996), His150, specific for *Clostridium perfringens/histolyticum* (Franks *et al.*, 1998), Lab158, specific for lactobacilli/enterococci (Harmsen *et al.*, 2000) and Rfla729, specific for *Ruminococcus flavefaciens* subcluster (Harmsen *et al.*, 2002).

4.3.4. Flow cytometry

A problem with the above mentioned molecular methods is that often a few bacterial species are present in such abundance that it is extremely difficult to obtain genetic information from rare microorganisms, and rRNA gene fragments of less-abundant microorganisms tend to be under-represented in PCR amplification (Muyzer *et al.*, 1993; Head *et al.*, 1998).

Flow cytometry (FCM) is an extremely versatile tool that can provide supplementary information on microbial ecology studies (Mason *et al.*, 1998). Flow cytometry used in conjunction with fluorescence-activated cell sorting can quantify and fractionate complex bacterial communities, and typically 102–103 cells/s can be characterised and sorted by flow cytometry (Davey and Kell, 1996; Porter *et al.*, 1996). The information that can be obtained using flow cytometry concerning individual microbial cells includes size, shape, surface texture, viability, DNA content (Robertson and Button, 1989), and specific staining conferred by fluorescent antibodies (Schönhuber *et al.*, 1997) or rRNA-targeted oligonucleotide probes (Wallner *et al.*, 1996; Zoetendal *et al.*, 2002). Microbial cell viability remains intact when dyes targeting cell surface molecules are used in FCM. This permits cell cultivation after FCM. Table 4 summarises the uses and limitations of some molecular techniques for microbiota identification.

5. Interactions between nutrition and gut microbiota. Results using molecular techniques

As was mentioned, the molecular techniques enhance the characterisation of gut microbiota with respect to cultivation ones, and could be a useful tool to understand the complex mechanisms that maintain the equilibrium among intestinal bacteria and the role of nutrition in the manipulation of gut microbiota. The information generated from these techniques is enormous because of the possibility to characterise the whole of the microbial structure and its individual composition (genera, specie and strains). Techniques such as DGGE and RFLP have been used for monitoring gastrointestinal microbiota in different animals. Based on the results obtained, several indexes have been proposed to classify this information in order to understand the factors that explain the development of a healthy population. The **biodiversity** and the **degree of similarity** give us general information about of the structure of the population, including that about known and unknown bacteria. The **biodiversity** index represents the number of sequences identified in the molecular data base that contain information either of known or unknown bacteria and so give us a relative value of the number of different bacteria in a population. The degree **of similarity** is based upon the determination of mathematical distances between two populations taking into account the type of bacteria and their relative quantity. This index permits to establish the phylogenetic relationships among bacteria and between microbial populations.

Studies based on these indexes have contributed increasing the knowledge concerning factors that affect microbial community structure, such as environmental perturbations, physiological conditions, gut localisation, and genetic background of the host in pigs, poultry, cattle, rodents and humans (Zoetendal *et al.*, 2004). Also in rabbits, the

Table 4. Advantages and disadvantages of molecular techniques in the microbiota characterisation.

Method	Advantages	Disadvantages
Direct culture	Detect some viable bacteria. Quantitative evaluation of some bacterial species.	Only cultivable bacteria can be analysed. The unviable and the uncultured bacteria are outside of the analysis.
RFLP	Detect cultivable and uncultivable bacteria. Useful for monitoring community structure. It uses a reduced number of primers. It is possible to correlate bands with some bacterial species. The information of the Ribosomal Database Project could directly be used to deduce the fragments expected from a specific bacterial species.	Bacterial identification requires clone library The absence of a band is related with the absence of some bacterial species, but the presence of a band is not always correlated with the presence of a specific bacterium. Time consuming for identification. Expensive.
DGGE	Detect cultivable and uncultivable bacteria. Useful for monitoring community structure It uses a reduced number of primers. It not uses restriction enzymes. It is possible to correlate some band position with some bacterial species.	For bacterial identification requires clone library. The information of the Ribosomal Database Project is not useful and it's necessary to construct a new database with the position of the bands obtained from different bacterial species. Errors in the identification of different strains from the same bacterial species, two ore more bands could be correlated with the same bacteria. Time consuming for identification.
Ribotyping	A single rRNA probe can be used to type all bacteria. Very reproducible and effective for the analysis of the intestinal microbiota.	Only cultivable bacteria are possible to analyse. The unviable and the uncultured bacteria are outside of the analysis. Time consuming.
Ribosomal DNA sequencing	Detect cultivable and uncultivable bacteria. The information of the Ribosomal Database Project could be used to confirm the presence of specific bacterial species or to detect new bacterial species.	Not possible to analyse this method a high number of samples. Time consuming. Expensive.
Direct amplification	Detect cultivable and uncultivable bacteria. Confirm the presence/absence of some bacterial species. Quantitative evaluation of some bacterial species.	For each bacterial species a tandem of primers is necessary (i.e. needs a high number of primers). Time consuming. Expensive.
Genetic probes	Detect cultivable and uncultivable bacteria. Confirm the presence/absence of some bacterial species. Semi-quantitative evaluation of some bacterial species.	For each bacterial species a specific molecular probes is necessary. Expensive.
Flow cytometry	Detect cultivable and uncultivable bacteria. Confirm the presence/absence of some bacterial species. Quantitative evaluation of some bacterial species. Rapid and automated analysis of mixed microbial communities.	For each bacterial species a specific molecular probes is necessary. Expensive.

use of RFLP technique has led to good results in the characterisation of microbiota at different gut locations (ileum vs caecum) or differences in the genetic of the animal. The results obtained by Pérez de Rozas *et al.* (2004) and Nicodemus *et al.* (2004) showed differences in bacterial community among species (pigs, poultry and rabbits) and also between different places in the gut in the same species. Ileum and caecum only showed a 65 % of **similarity** among bacteria community in rabbits. Consequently, the studies of factors that affect intestinal health could give different results depending on the place where the microbial sample has been taken. As occurs in humans using twins and unrelated persons, the genetic characteristics of the host could have an important influence in the predominant microbiota. Humans' studies show that similarity is significantly higher in twins than in unrelated persons (Zoetendal *et al.*, 2001). According to García *et al.* (2005), the degree of similarity of caecal microbiota between the mother and its corresponding litter may be very low (only 10% between the mother and the young rabbits at 16 days of age) possibly due to differences in the food ingested (solid food vs milk). However, when young rabbits from different litters are compared, the brothers present the maximum of similarity (47%). These findings agree with the great influence of the litter origin in the incidence of digestive pathologies in the growth trials. Once again the importance of taking into account the litter in the design of the nutrition experiments is highlighted to distinguish the effects of the host and of the diet on the microbial ecosystem.

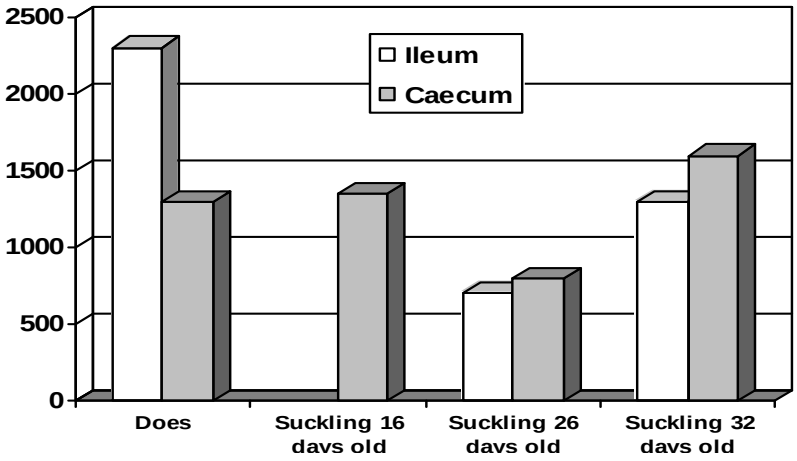


Figure3. *Biodiversity of ileal and caecal samples in does and their litters at different ages.*

The **biodiversity index** has been used to inform about the stability of microbial ecosystem in human experiments. Healthy adult humans show high stability; however, the biodiversity shifts in elderly or newborn babies. In rabbits, the biodiversity changes with the age and with the weaning. According to García *et al.* (2005), suckling rabbits at 16 days of age present a caecal biodiversity similar to their mother, but when the rabbits begin to eat solid feed the biodiversity decreased. After that it increased analogous to solid feed intake. The weaning would also produce a decrease (from 2000 to 600 identified sequences) in the caecal biodiversity (Figure 3). The interpretation of these findings in terms of stability or intestinal health security is not easy and should be complemented by more precise information about the changes produced in the commensal bacteria and potential pathogens. As was mentioned earlier the doe and the litter at day 16 of lactation had the same biodiversity but the degree of similarity of ceacal microbiota was only 10%.

The same analysis also gives a qualitative information about the presence or absence of known or unknown bacteria by comparision with the molecular data bases and permit to investigate the effect of different factors on a particular bacterial genera or specie, commensal or pathogen. Taking into account all the information, it permits to relate both structural and particular caracteristics of a microbial population. This information is very useful especially in nutrition experiments where the interaction between diet and commensal and/or pathogenic bacteria could be important.

In a series of three experiments, between microbiologist and nutritionist (CRESA, Barcelona and UPM Madrid), the RFLP technique was used to study the effect of nutrition on intestinal microbiota, in a context of a farm with Epizootic Rabbit Enteropathy. The dietary factors studied were those most related with the digestive problems in rabbits: the level and type of fibre and protein (Nicodemus *et al.*, 2004; Goméz-Conde *et al.*, 2004; Chamorro *et al.*, 2005; Gómez-Conde *et al.*, 2006). All these experiments were performed using healthy animals, weaned at 25 days and supplemented with antibiotics (Zn bacitracin and Apramicine) or without any antibiotic. The main results are presented in the Table 5.

The lowest mortality was observed with dietary levels of insoluble fibre (NDF) of 30%, 12% of soluble fibre (by the inclusion of beet pulp) and 16% of protein, with respect to diets with higher levels of protein or lower levels of NFD or the inclusion of fibre with low level of soluble fibre. Except for the study of dietary fibre level (Nicodemus *et al.*, 2004), the reduction of mortality occurred parallel to a reduction in the proportion of animals where

Table 5. Effect of type of diet on Biodiversity, the proportion of animals where potential pathogenic bacteria were detected and on the mortality in rabbits.

	Biodiversity		*Clostridium perfringens*		Other bacteria		Mortality
	Ileum	Caecum	Ileum	Caecum	Ileum	Caecum	
Decrease in the level of fibre (25 vs 30% NDF) [(1)]	Increase	Decrease	No effect	No effect	Increment of bacteroides	Decrease of *Bacteroides* and *Ruminococos*	Increase
Increase of particle size (large vs normal) [(1)]	Decrease	Decrease, low fibre diets (25 % NDF)	No effect	No effect	Decrease of *E. coli*, *Helicobacter* or *Yersinia*	Little effect	No effect
Increase of soluble fibre [(2)]	No effect	No effect	No effect	Decrease	Decrease *Campylobacter*	Decrease *Campylobacter*	Decrease
Increased level of protein (16 vs 18% CP) [(3)]	Decrease	No effect	Decrease	No effect	Decrease *Campylobacter*		Decrease
Type of protein (alfalfa vs soybean concentrate + fibre) [(3)]	No effect	No effect	No effect	No effect	Decrease *Clostridium s*pp.		No effect

[1]: Nicodemus *et al.*(2004) [2]: Gómez- Conde *et al.* (2004, 2006) [3]: Chamorro *et al.* (2005)

Clostridium perfringens was detected. A toxin produced by a strain of *Clostridium perfringens* has been associated to the mortality produced by the Epizootic Rabbit Enteropathy (Pérez de Rozas *et al*, 2005). However, this effect was more evident in ileal (protein level) or caecal samples (type of fibre) depending on the type of substrate available to microbiota.

Same opportunistic harmful bacteria associated with the intestinal mucosa, such as *E. coli*, *Campylobacter*, *Yersinia* or *Helicobacter* seem to be sensitive to changes in the diet when the sample is taken at the ileum than in the caecum. So a correct selection of the sampling intestinal place is important to detect significant effects of the diet on potential pathogenic bacteria.

With respect to the microbiota structure, a less **biodiverse** microbiota seems to favour a higher proliferation of *Clostridium perfringens* in piglets. Pigs parenterally nourished seem to favour a higher proliferation of *C. perfringens* with respect to those fed enterally. This tendency has not been confirmed in rabbits fed enterally with several diets. Increments of the animals with presence of *C. perfringens* are observed parallel to the increments in the biodiversity (Chamorro *et al.*, 2005) or without changes in this index (Gómez Conde *et al.*, 2006). However, ileal biodiversity seem to be more correlated with mortality. An increment of ileal biodiversity leads to higher mortality. So this index could be used as a predictor of the intestinal health if these results are confirmed.

The results confirmed the power of these techniques to study the role of nutrition in modulating the intestinal microbiota in rabbits. However, it is important to take into account the high number of experimental units required to detect significant differences using these techniques and apparently healthy animals. In this situation, the proportion of animals with presence of identified bacteria is low and the biodiversity presents a high individual variability. A minimum of 20 animals per treatment, blocked by litter, is recommended.

Another alternative to diminish this problem is to perform the nutrition experiments using infected animals with the pathogen, as has been used in pigs or poultry. The main disadvantages of this technique are the high biosecurity measurements required, only possible in special farms, and the extrapolation of these results to practical conditions where interactions between the pathogen and commensal bacteria could change the results.

6. Indirect measurements of microbial activity in the gastrointestinal tract used in nutrition experiments

Similar to other herbivorous animals, the rabbit caecal ecosystem hydrolyses and ferments a large variety of dietary components. The measurement of this microbial activity is essential to estimate the

digestive efficacy of the flora but is also an alternative to evaluate relationship with digestive health.

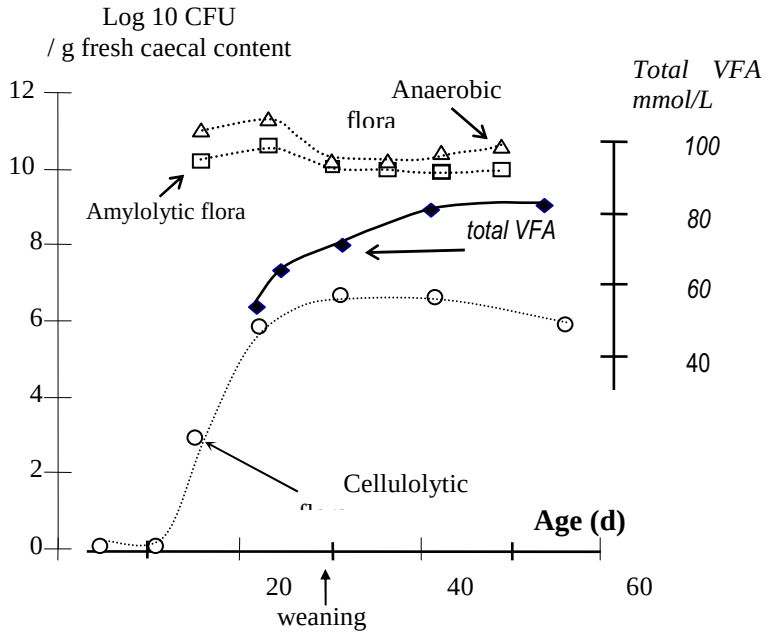
Historically, a fermentative activity was first identified in the rabbit caecum (Elsden *et al.*, 1946), and then the cellulolytic activity of the rabbit caecal flora was evidenced in-vitro by Cools and Jeuniaux (1961). The caecal metabolism of nutrients is similar to that of other herbivores but exhibits some particularities. The caecal VFA profile is specific to the rabbit, with a predominance of acetate (C2 = 60 to 80 mmol/100 mol) followed by butyrate (C4 = 8 to 20) and then by propionate (C3 = 3 to 10). Moreover, Adjiri *et al.* (1992) showed *in vitro* (using a semi-continuous flow fermentor) that this VFA pattern was specific to the caecal flora and not to the composition of the fermented substrate. The fermentative activity of the bacteria varies according to the circadian rhythm of the intake behaviour (subchapter 4.1), including a lower concentration of VFA (-25%) during the caecotrophy period compared to a higher VFA level found during the hard faeces excretion phase (Gidenne, 1986; Bellier *et al.*, 1995; Bellier and Gidenne, 1996). This diurnal pattern of the caecal fermentative activity coincides with a similar rhythm of VFA absorption and metabolisation (Vernay, 1989).

Studies on caecal fermentative activity are usually restricted to ammonia and VFA caecal concentrations measurements. Almost no results are available on VFA or gas production (hydrogen, methane) associated with fermentation, except an *in vitro* study evaluating that methanogenic activity was around zero until weaning, and rose after 36 d of age (Piattoni *et al.*, 1996). Besides, the caecal fermentative activity evolves sharply with age, according to the composition of the meal: in exclusively milk-fed rabbits (till 15d old) the caecal VFA level remains very low (<10 mmol/L, Padilha *et al.*, 1995) and then rises with the intake of solid food up to 60-80 mmol/l at 6 weeks of age (Figure 4).

Studies in digestive microbiology were also performed to estimate the colonisation rate of a specified substrate by caecal bacteria, in in-vitro conditions (e.g. roll tubes techniques). For instance, Boulahrouf *et al.* (1991) have shown that caecal bacteria are able to grow on cellulose paper substrate or citrus pectin or beechwood xylan. Similarly, ureolytic proteolytic and amylolytic bacteria were isolated from the caecum and caecotrophes (Crociani *et al.* 1984; Emaldi *et al.*, 1979; Padilha *et al.*, 1995). However, this approach is time consuming, and it remains difficult to follow the implantation dynamic or relationship with nutrient intake. Furthermore, only cultivable bacteria are identified.

More recent studies have dealt with two other approaches for evaluating the microbial activity in digestive ecosystems. The measurement of the ATP level as an indicator of the energetic metabolism of the bacteria was validated in pigs (Bach Knudsen *et al.*, 1991), but showed too-high inter-individual variability to be of interest for studies in the rabbit (Bellier and Gidenne, 1996). The evaluation of the microbial biomass using an internal marker of bacteria, such as DAPA (diaminopimelic acid), was initially developed for ruminants. A recent application of this method in the rabbit indicated a good relationship between biomass produced and fibre intake (Jehl and Gidenne, 1996; Gidenne *et al.*, 2004). Further studies are required to confirm the validity of this approach; however these techniques are time-consuming and costly.

Another approach consists of evaluating the hydrolysing capacity of the flora by extracting their enzymes and assaying them in-vitro on purified substrates. This method was first developed in ruminant animals and then adapted to the rabbit caecal ecosystem in 1995 (Jehl *et al.*, 1995). Although lower compared to the rumen, the caecal fibrolytic activity is relatively high for pectins (Marounek *et al.*, 1995; Gidenne *et al.*, 2002) and



(adapted from : Boulahrouf *et al.*, 1991; Piattoni *et al.*, 1995; Padilha *et al.*, 1995)

Figure 4. *Kinetics of establishment of the caecal flora and of volatile fatty acids (VFA) in the growing rabbit.*

hemicelluloses (xylane) and lower for cellulose (either carboxymethyl-cellulose or avicellose). Besides, the bacterial fibrolytic activity is similar in caecum and soft faeces (Jehl *et al.*, 1996), so it is possible to follow the bacterial fibrolytic activity of one individual without cannulation techniques. The fibrolytic potential of the caecal flora seemed to evolve weakly between 5 and 10 weeks of age (Gidenne *et al.*, 2002), but seemed higher in the adult animal (Pinheiro *et al.*, 2001). However, these enzymatic parameters remain relatively variable, and a high number of measurements is necessary.

The caecal microbial activity can also be addressed through its contribution to the supply of nutrient via the ingestion of soft faeces. Piattoni *et al.* (1995) evaluate that around 50% of the caecal nitrogen was of bacterial origin, using a new indicator based on RNA/crude protein ratio. The nitrogen produced by the flora and recycled in soft faeces was evaluated recently using a method based on the measurements of the purine/bacterial nitrogen ratio (Garcia *et al.*, 1995) or on the measurements of DAPA levels (Jehl and Gidenne, 1996), in isolated caecal bacterial preparation. According to this technique, the bacterial contribution to the total nitrogen intake ranged between 12 and 24%. More recently, new methods were developed to more precisely assess the nitrogen metabolism and the impact of the caecal flora, using either purines derivatives (Balcells *et al.*, 1998) or labelled nitrogen sources (NH_4Cl-N_{15}; Bellenguer *et al.*, 2005).

Interaction among nutrition and caecal microbial activity was subjected to numerous studies in the growing rabbit. A large number of authors deals with the effect of the fibre intake on microbial activity and the relationship with the digestive health of the rabbit, since a too low fibre intake increases the incidence of digestive disorders (see subchapter 4.3).

Increasing the fibre intake (and lowering that of starch) either increase or has no effect on the fibrolytic activity and caecal VFA concentration, while a lower butyrate molar proportion is generally registered. The quality of fibre, particularly their fermentability, is able to modulate the microbial activity. For instance, increasing the intake of pectins or hemicelluloses generally stimulates the flora activity (Garcia *et al.*, 2000; Gidenne and Bellier, 2000; Gidenne *et al.*, 2004). In a collaborative study, Garcia *et al.* (2002) reported that dietary uronic acids concentration is positively correlated to the caecal VFA and propionic levels. In association to changes in microbial activity it is suspected that nutrition also modulates the microbial population balance, as suggested by Belenguer *et al.* (2000).

7. Conclusions

The gastrointestinal microbiota is a complex community that remains largely unknown. The description of the pathogens, their mechanism of pathogenicity and their interaction with commensal bacteria is necessary to elaborate funded strategies for the prevention of intestinal disorders. The colonisation of intestine is age dependent and can be modulate with the diet. Indirect measurement of microbial metabolic activity has been a useful tool in studying the role of nutrition in the growth of microbial community. However, for a fine tuning of the microbiota, the use of the new molecular techniques could provide a better knowledge about the role of individual bacteria in the community to establish a healthy microbiota

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4.3. Nutritional strategies improving the digestive health of the weaned rabbit

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1. Introduction

The digestive health covers all the parameters enabling the animal to maintain its intestinal equilibrium, in response to various factors such nutrients intake or exogenous microorganisms. If the digestive balance is not maintained, troubles could appear such as diarrhoea in the young animal, either because of gut colonisation by an identified pathogen (e.g. *E. Coli*) or from a multifactorial origin. However, within a group, animals differently develop the clinical symptoms (diarrhoea, impaction) and not all the sick animals die. Several mechanisms of defence could explain the variability in the disease sensibility such as: the gut barrier function, the competitive exclusion between saprophyte and pathogen bacteria and the immune status. Nutrition and feeding strategies also play an important role in digestive health, in supplying the adequate nutrients quantity and quality, to improve: i) mucosa integrity and immune response (avoiding pathogen attachment and colonisation) ii) the growth/stability of the commensal microbiota (barrier effect).

To develop accurate nutritional strategies it is necessary to identify the specific nutrients or bioactive components in feeds (or milk) that enhance these mechanisms of defence. It is also essential to know what nutrients are digested before the ileum and what nutrients constitute the substrate for caecal microbiota. These nutritional strategies must be focused around the weaning period, since it is a critical phase for sensibility to digestive diseases, probably linked to the processes of digestive maturation, including the development of microbiota (see 4.2) and the immune system (see subchapter 4.1 section 3). Besides, the strategies relating to the unweaned or early-weaned rabbit (<26d old) will be discussed in the subchapter 4.4.

2. Statistical parameters to evaluate digestive health and risk factor

The traditional indicator to evaluate the impact of a disease in rabbit breeding is the mortality rate. Recently, a morbidity indicator was developed to assess more precisely the incidence of the clinical symptoms (Gidenne, 1995), and it could be combined with mortality to obtain the health risk index ("HRi"= morbidity + mortality rate). This approach allows a more precise assessment of the health status. But, these traits show large variations according to many factors. For instance, mortality rate of rabbits fed the same diet could range from 0 up to 70% according to various factors, such as: litter effect, preventive medication, age at weaning. Thus it means that a large number of animals is required to detect a significant difference between two treatments in mortality. For instance, to detect a 5% deviation among two mortality rates, more than 300 animals are required in each group (Table1).

When the clinical symptoms (diarrhoea, caecal impaction, stomachal borborigmus …) are clear, the morbidity rate is relatively easy to measure. However, when only a reduction of growth rate is detectable, a threshold must be defined to class the animal as morbid or not, such as the average minus 2x standard deviation (signifying the 2.5% of the animals with lower growth rate), or up to 3 SD. But it requires a large set of rabbits within a group to

define precisely the mean and its range of variation. Moreover, it must be outlined that adequate statistical methods are necessary to treat discrete data (such mortality or morbidity). For instance, when analysing models with more than one factor or including more than two levels (within a factor) or to test interaction among two factors, specific categorical analysis based on a weighted least square analysis must be used instead of a simple Chi² test.

Table 1. Number of rabbits per treatment required (n) to detect a significant difference (P = 0.05) for mortality rate between two treatments.

Difference to be detected, percentage units	Number of rabbits required (n)
5	338
10	87
15	40
20	23

3. Interaction between nutrient intake and digestive health of the weaned rabbit.

3.1. Fibre and starch intake

Many experiments have been performed to elicit the respective effects of fibre and starch on the incidence of diarrhoea in the growing rabbit, particularly just after the weaning (Colin *et al.*, 1976; De Blas *et al.*, 1986; Blas *et al.*, 1994; Bennegadi *et al.*, 2001). This period is critical since there is a large incidence of digestive troubles, and also because an active digestive maturation is occurring and feed intake is increasing sharply. An increased dietary starch/fibre ratio (<30% NDF, <15% ADF, >20% starch) could lead to both a lower ileal flow of dry matter and bacterial biomass production in the caecum of the young rabbit, which is often associated with a lower fermentative activity (higher pH), modified fermentation pattern (higher butyrate proportions) and lower fibrolytic activity of bacteria (Bellier and Gidenne, 1996; Gidenne *et al.* 2000, 2002, 2004a; Nicodemus *et al.*, 2003a, 2004).

However, it still remains difficult to explain how these changes in caecal digestive processes determine the greater incidence of digestive troubles (diarrhoea mainly) observed with low fibre diets.Probably the microbial ecosystem is largely affected, such the archaeal community which, was twofold higher with standard diet than with fibre-deficient (Bennegadi *et al.*, 2003). Furthermore, when dietary NDF decreased from 30 to 25% microbiota biodiversity increased at ileum but was reduced at the caecum (Nicodemus et al,. 2004). Besides, the favourable effect of a high fibre intake on rabbit digestive health was also shown using experimental infection model reproducing a colibacillosis (Gidenne and Licois, 2005, see section 3.2).

However, experiments that dealt with this question compared diets having varying levels of fibre and simultaneously an inverse variation of the starch level (since rabbits are a complete pelleted feed). Consequently, when a study reported a positive effect of an increased dietary fibre intake on digestive health, it was in fact difficult to exclude that there was also an effect of a reduced starch intake. We thus have to deal with two opposite hypotheses: are digestive troubles linked to a carbohydrate overload in the caecum or linked to a fibre deficiency (or both) ?. Recently, this question was elicited by studying the ileal flow of starch and fibre in the growing rabbit (5-9 wk. old). With high starch diets (≥30% starch mainly from wheat) the ileal starch digestibility was very high (>97%), the flow of starch remained under 2g/d (intake ≈ 30g/d) at ileum, while that of fibre was at least 10 times higher (≈ 20g NDF/d) (Gidenne *et al.*, 2000; Nicodemus *et al.*, 2004; Garcia *et al.*, 2004a). Thus an overload of starch seems very unlikely since starch digestion was very efficient already at 5 wk. old. Moreover, a large-scale study using a network of 6 experimental breeding unit (GEC French group) demonstrated through a 2 x 2 factorial design (two level of starch "12 vs 19%" combined with two ADF levels "15 vs 19%") that only the fibre level play a role in digestive trouble occurrence, and not the starch level (Gidenne *et al.*, 2004b). Furthermore, by comparing iso-fibre diets but with several starch sources varying in their intestinal digestion (maize, wheat, barley) Gidenne *et al.* (2005) observed no effect of starch ileal flow on diarrhoea incidence in the weaned rabbit. Fibre intake thus plays a major role in the determiniation of digestive trouble in the classically weaned rabbit (28-35 d old).

With earlier weaned rabbits (at 25d of age), Gutiérrez *et al.* (2002a) observed that mortality remained low and similar with diets having 36 vs 30% of NDF, but mortality rate tended to increase (P=0.06) after a feed shift (at 39 d of age, from experimental to commercial diet) for those previously fed with 36% NDF diet.

Accordingly, several large-scale studies aimed at validating clearly the relationship among dietary fibre/starch levels and diarrhoea incidence for the "classically" weaned rabbit, using experimental design with a high number of animals per treatment. The relationship between low fibre diets (<14% ADF) and a higher incidence of diarrhoea was clearly established in two studies where the quality of fibre, e.g. the proportions of fibre fraction as analysed through the Van-Soest procedure, was controlled (Blas *et al.*, 1994; Bennegadi *et al.*, 2001). In France several large-scale studies (using at least 300 animals per treatment and 5 sites) were performed to precify the fibre recommendations for

Table 2 Fibre and starch requirements for the young rabbit after weaning to prevent digestive troubles.

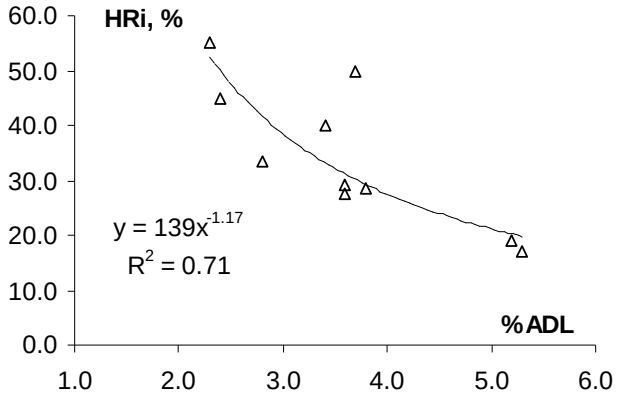
Unit[1]	INRA		Univ. Madrid	
	Post weaning (28-42d old)	Growing (42-70d old)	Post weaning (25-39d old)	Growing (39-70d old)
Neutral Detergent Fibre "NDF"	NDF≥310	NDF≥270	300≤NDF<360	320≤NDF<350
Lignocellulose "ADF"	≥190	≥170		160≤ADF<185
Lignins "ADL"	≥55	≥50		≥55
Cellulose "ADF-ADL"	≥130	≥110		
Ratio Lignins/ Cellulose	>0.40	>0.40		
Hemicelluloses "NDF-ADF"	>120	>100		
DgF^2/ADF	≤1.3	≤1.3		
Neutral detergent soluble fibre "NDSF"[3]	–	–	120	–
Particles > 0.3 mm	–	–	–	> 210
Starch			<200	145<starch<175

[1] g.kg-1 as fed basis, corrected to a dry matter content of 900 $g.kg^{-1}$.
[2] Digestible fibre fraction = [hemicelluloses (NDF-ADF) + water-insoluble pectins].
[3] According to Hall *et al.* (1997)

prevention of digestive troubles in the growing rabbit. The relevance of Van-Soest criteria was studied, since crude fibre method was too imprecise for this purpose. A review of these studies and of new fibre recommendations was recently published (Gidenne, 2003). We here proposed a summary of the fibre requirement (Table.2) for post-weaned and growing rabbits from French (INRA) and Spanish (Univ. Madrid) research groups.

3.2. Effects of the type of fibre

Apart from the important role of fibre intake, the quality of fibre also interferes with diarrhoea incidence in the growing rabbit (30-70 d old). The favourable effect of lignocellulose on the digestive disorders and mortality in fattening rabbits was evidenced by several studies. For example, the health risk index (HRi = mortality + morbidity rate) decreased from 28 to 18% when the dietary ADF content increased from 15 to 19% (Gidenne *et al.*, 2004b). However, within lignocellulosic components, the favourable effect of the lignin fraction (criterion ADL = Acid Detergent Lignin) was also demonstrated with several studies, and a strong negative relationship was found with the HRi (Figure 1, R^2=0.71; n=10 diets with ADF level from 14 to 20%).



ADL = Acid detergent lignin (Van-Soest sequential procedure, EGRAN 2001).

HRI = Health risk from digestive trouble = Mortality + morbidity rate by diarrhoea, measured from 28 to 70d of age with at least 40 rabbits/diet (Data for 10 diets ranging from **14 to 20% ADF** level; references: Gidenne, 2003).

Figure 1. *Reduction of digestive troubles incidence according to dietary lignin.*

Parallel with this, the intake of lignins involves a sharp reduction of the feed digestibility, associated with a reduction of the digesta retention time in the whole tract (-20%), and with a rise of the feed conversion ratio. The cellulose (ADF-ADL) also favours the digestive health. However, lignins play a specific role since an increase of the ratio lignins/cellulose (L/C) is associated with a lower HRi (Gidenne *et al.*, 2001a). Globally, the ADL

requirement for the growing rabbit can be assumed as to 5 to 7g/d, and that of cellulose from approximately 11 to 12 g/d. However, to date, no correct and quick analytical method for lignins is available. Consequently, estimating the amount of lignins in a raw material remains difficult, particularly in tannin-rich ingredients (grape marc, etc.), and caution must be taken to fit requirements.

Although digestive health of the classically weaned rabbit depends on the level and quality of lignocellulose, it also varies greatly for the same ADF level (Figure 2), because the level of more digestible fibre fractions "DgF", i.e. [hemicelluloses (NDF-ADF) + water-insoluble pectins], could also vary independently of lignin and cellulose levels. For instance the ratio DgF/ADF ranged from 0.9 to 1.7 in figure 2. The DgF fraction would play a key role for the digestive efficiency and health, since it is rapidly fermented (compared to ADF), in a time delay matching the retention time of the caeco-colic segment (9-13h, Gidenne, 1994). Without changes in ADF dietary level, digestive troubles are rather reduced when DgF replaces starch (Perez *et al.*, 2000) or protein (Gidenne *et al.*, 2001b). This could originate from the favourable effect of DgF (compared to starch or protein) on caecal fermentative activity (Garcia *et al.*, 2002), and possibly from their moderate effect on the rate of passage (Gidenne *et al.*, 2004b). However, a too high incorporation of DgF with respect to lignins and cellulose should be avoided to minimise the Health risk during fattening. It is thus recommended that the ratio DgF/ADF remains under 1.3 (when dietary ADF level is over 15%, see Table.2).

Another way to analyse the role of cell-wall polysaccharides that are rapidly fermented is to determine the NDSF residue (Hall *et al.*, 1997), which corresponds to the cell wall polysaccharides soluble in neutral detergent solution (= sum of water soluble and insoluble pectins + β-glucans + fructans + oligosaccharides[DP>15]). Although the level of NDSF is moderate in rabbit feeds, a reduction of its level (12% vs 8%) could be unfavourable on digestive health of the early-weaned rabbit (Gómez-Conde *et al.* 2004b and 2005; Table 3). Reversely, a higher level of NDSF improved the mucosal morphology and functionality and its immune response. Besides, soluble fibre reduced the proportion of animals with Clostridium perfringens in the caecum and other pathogens as Campylobacter both in the ileum and in the caecum. Accordingly, mortality due to REE was reduced with a diet with 12% soluble fibre (Table 3; Gómez-Conde *et al.*, 2004a,b, 2005, 2006).

The favourable effect of dietary fibre was also recently analysed in the young during the weaning period (3 to 5 wk. old) by Fortun-Lamothe et al.

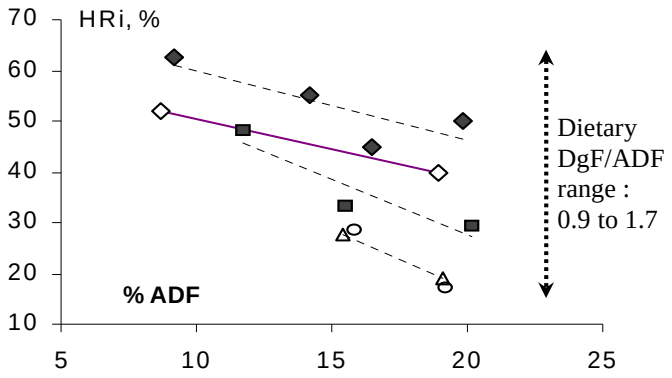


Figure 2. *The risk of digestive trouble (HRi) in the growing rabbit is jointly dependent of low-digested "ADF" and digestible fibre "DgF".*

ADF = lignocellulose (Van-Soest sequential procedure, EGRAN 2001).
DgF: digestible fibre = water insoluble pectins + hemicelluloses (NDF-ADF)
HRi= Health Risk index from digestive trouble = Mortality + morbidity rate by diarrhoea, measured from 28 to 70d of age, on at least 40 rabbits/diet (one point = one diet, n=13; for references see Gidenne 2003).

Table 3. Effect of dietary NDSF level on digestive parameters at 35d, and mortality in 25d weaned rabbits.

Dietary NDSF[1] level, % (as fed)	12	9	7	P level
Jejunum morphology and functionality (35d)				
Villi length, µm	721^{a}	567^{b}	492^{c}	0.05
Crypt depth, µm	89^{b}	115^{a}	113^{a}	0.05
Saccharidase activity (U/mg tissue)	8500^{a}	7100^{b}	5400^{c}	0.05
Immune response in *lamina propria* (35d)				
CD4+, %	35	33	26	NS
CD8+, %	21^{b}	27^{b}	31^{a}	0.05
C. perfringens, %[2]	8^{b}	6^{b}	19^{a}	0.05
Mortality 25-60 d, %	5,3^{b}	8,5^{ab}	14,4^{a}	0.05

[1] Neutral detergent soluble fibre according to Hall *et al.* (1997) [2] Frequency of detection in the ileum or caecum

(2005) in a large-scale study (6 sites + 3 reproductive cycles). They reported a lower mortality rate for litters fed a diet rich in fibre or when fibre+lipids replaced starch. Optimisation of the feeding strategies for doe and litters is presented in section 4.5. Besides, the favourable effect of fibre intake on rabbit digestive health was also shown using experimental infection model reproducing a specific pathology, such colibacillosis or REE (see section 4.2.5). The resistance of rabbits challenged with an experimental inoculation of enteropathogenic *E. Coli* was better when fed a high fibre diet (Gidenne and Licois, 2005). Similarly, the resistance of growing rabbits faced with spontaneous enterocolitis (REE) was better when fed a diet with a high ratio fibre/protein (Gidenne *et al.*, 2001b).

Dietary fibre quality could also be completed by the determination of the particle size pattern. It is acknowledged that the particle size distribution of a feed could affect the digestive motility and more particularly the cæco-colic rate of passage. Fibrous raw materials with a small proportion of large particles (> 0.3 mm) due to grinding (screen size 0.5 to 1 mm) or to a previous processing lead to longer retention time (Laplace and Lebas, 1977; Gidenne *et al.*, 1991; García *et al.*, 1999), but are not associated with a negative effect on the digestive health status (Lebas *et al.*, 1986; Gidenne *et al.*, 1991, Nicodemus *et al.*, 2006). Only a very low rate of large particles (< 21% particles lower than 0.3 mm) would have a negative impact on the performances. Nevertheless, a rate of coarse particles lower than 25 % is unusual in practice, since out of a series of 77 commercial French feeds the average proportion of coarse particles is 38.8% (minimum = 22.7%, mean minus 2 S.D. = 27%; Lebas and Lamboley, 1999).

In conclusion, one criterion is not sufficient for fibre recommendation, since the risk of digestive trouble in the growing rabbit is jointly dependent of low-digested "ADF" and digestible fibre "DgF". In perspective fibre role for the young rabbit should be precised, particularly the effects of NDSF fibre fraction.

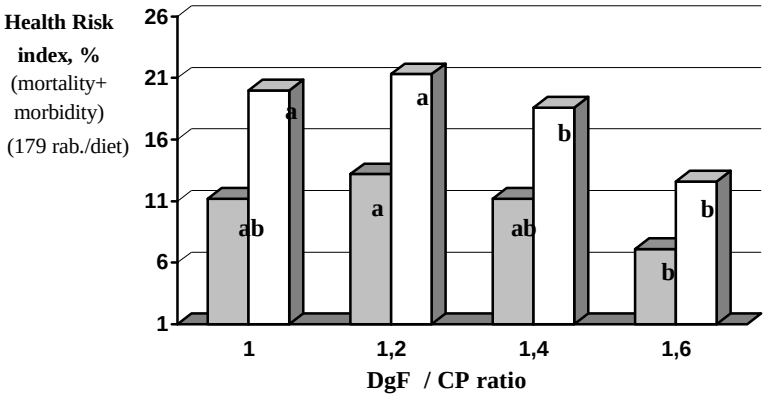


Figure 3. *Replacement of digestible fibre "DgF" by protein "CP": impact on the digestive health between weaning and slaughter.*

3.3. Effects of non structural carbohydrates (starch, lactose).

The starch quality impact depend sharply on the ileal starch flow, more variable in young compared to adult rabbit. Higher ileal concentrations of starch are found when pea or potato is incorporated in the diet compared to wheat or barley, both in growing (Blas *et al.*, 1994; Pinheiro et Gidenne, 2000; Gutiérrez *et al.*, 2002b) or in adult animals (Gidenne and Perez, 1993). A dietary supplementation with enzymes (β-glucanases, β-xylanases, α-amilases and pectinases. Porzyme 100®) reduced ileal starch concentration and mortality (Gutiérrez *et al.*, 2002b; Cachaldora *et al.* 2004). However, a substitution of pea by wheat and the heat treatment of these starch sources reduced ileal starch concentration but did not reduce mortality. Likewise, Gidenne *et al.* (2005), by comparing iso-fibre diets (29% NDF) but with several starch sources varying in their intestinal digestion (maize, wheat, barley) observed no effect of ileal starch concentration on diarrhoea incidence in the weaned rabbit. These results also support the weak influence of starch on the digestive health when fibre requirements are covered. The positive effect of enzyme supplementation might thus be related with the partial hydrolysis of non-starch polysaccharides that produce complex oligomers, which might modulate gut microbiota and lead to a better digestive health.

Since starch digestion is incomplete in the young rabbit, replacement of some starch by lactose has been studied, as occurs in piglets' diets. However, lactose ileal digestibility was much lower than that recorded for starch (74 vs 92%), which might be due to the severe reduction of lactase activity after weaning. This result led to a higher ileal flux of lactose and a higher mortality (Gutiérrez *et al.*, 2002a), possibly explained by a flora unbalance in the caecum.

3.4. Effects of protein level and quality

Protein requirements are high in young animals not only for body growth, but also for intestinal mucosa development and renewing. The replacement of fibre by protein thus corresponding to a lower PD/DE ratio would influence the digestive health after weaning, following a curvilinear relationship (De Blas, 1981). Similarly, an excessive replacement of digestible fibre by protein increases the health risk for diarrhoea, as is shown in a large scale study (Figure 3; Gidenne *et al.*, 2001b). A hypothesis to explain it could be a higher availability of substrates for microbial growth, with a prevalence of pathogenic species,

when animals are fed with high protein diet. Accordingly, a higher ileal flux of protein increased the caecal acidity (Figure 4). (Gutiérrez *et al.*, 2003; Nicodemus *et al.*, 2003b, 2004; Gómez-Conde *et al.*, 2004a,b and personal comm.), and may favour pathogenic species. This event would be more important in young rabbits due to the unachieved digestive maturation. Besides, some harmful genera, as *E. Coli* or Clostridia, can use amino acids as substrate for growth. For instance, Clostridium increased when the diet contained an excess of protein (Catala and Bonnafous, 1979; Haffar *et al.*, 1988).

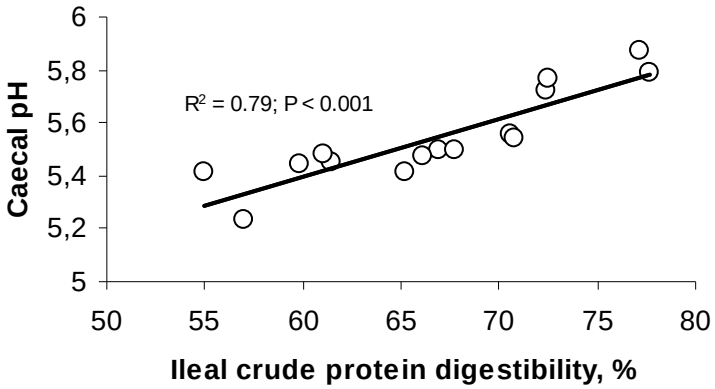


Figure 4. *Effect of apparent ileal digestibility of crude protein on caecal pH of 35d old rabbits (n = 15), weaned at 25d.*

Weaning also implies a switch from milk to vegetal proteins. The latter are less digestible and sometimes contain antinutritive factors, such as lectins, antitrypsic or antigenic factors. This could impair the apparent ileal digestion or induce changes in the morphology of intestinal mucosa as occurs in other species. In rabbits, Scheele and Bolder (1987) observed an increase of mortality before weaning (35 d of age) in rabbits fed diets containing a high proportion of soybean meal (20%) with respect to diets based on animal protein (31 vs 10%, respectively). In this sense, Gutiérrez *et al.* (2000) observed that the substitution of soybean meal with animal plasma had a positive effect on the morphology of intestinal mucosa, feed intake, growth and mortality.

In another study, Gutiérrez *et al.* (2003) compared four protein concentrates (sunflower meal, soybean meal 48, soybean concentrate and potato protein) in isonutritive starter diets. Animals fed diets with the protein sources with lesser content in antinutritive factors (sunflower meal and soybean concentrate) showed higher apparent ileal protein digestibility and growth performance and lower mortality rate than the other diets (Figure 5). However, the gastric acidity, villus morphology and faecal digestibility were similar among diets, and no differences on phenotypic distribution of lymphocytes in the duodenal *lamina propria* were detected, which might be related to the development of a tolerance mechanism by the animals. The importance of the reduction of the protein flux in the ileum (by using digestible sources or reducing protein level) in reducing the mortality rate has also been supported in recent experiments (García *et al.*, 2004b; Chamorro *et al.*, 2005). In these studies the digestible protein levels were high and covered the essential amino acids needs, so no dietary effects were observed on intestinal mucosa.

Besides, the protein intake could also affect the small intestinal mucosal integrity and function via a modulation of the local immune responses and possibly implying inflammation reactions, as shown in piglets (Vente-Spreeuwenberg *et al.*, 2004). However, as faras we know, very little and partial works has been carried out on rabbits on this topic, and furthermore no differentiate protein sources are used in diets for weaning or older rabbits. Gutiérrez *et al.* (2000) observed that inclusion of animal plasma instead of soybean meal improved intestinal mucosal morphology in early weaned rabbits, whereas other studies did not find differences in villi height and

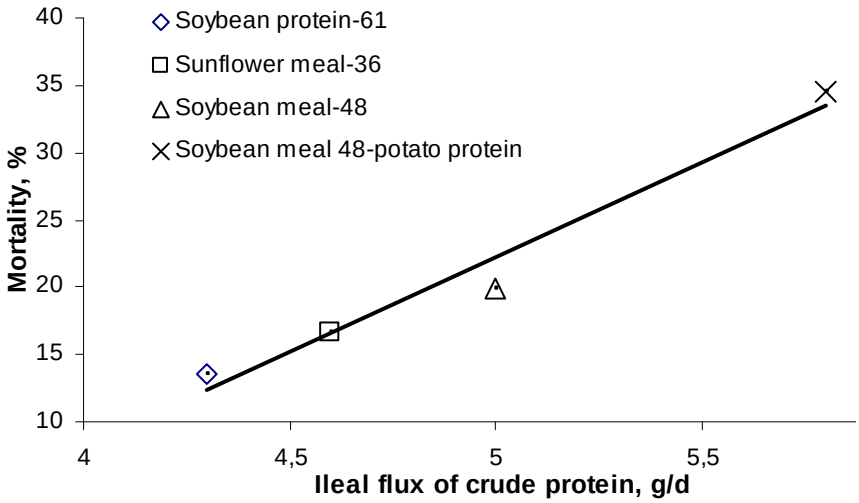


Figure 5. *Effect of ileal flux of protein on mortality from 25 to 60 d of age.*

crypt depth (Gutiérrez *et al.*, 2003) or in phenotypic distribution of lymphocytes in the duodenal *lamina propria* (Campín *et al.*, 2003) when including different protein sources. Cano *et al.* (2004) reported that rabbits fed a soybean meal-rich diet had a lower feed intake around weaning, associated with a higher serum anti-feed IgG. It may be hypothesised that it provokes a sub-chronic inflammation process, and that may increase the sensibility of the young rabbit to digestive diseases.

3.5. Effects of lipid level and quality

Few studies dealt with the role of dietary lipids on digestive health of the growing rabbit, since lipids dietary levels are usually under 3% and are well digested in the small intestine. Furthermore, it is difficult to separate the effect of lipids itself from that of DE intake. However, it has been recently found that some medium-chain fatty acids, such as caprylic and capric acid (as triacylglycerol form), exhibit antimicrobial activity for some bacteria of the caecal digestive flora (Marounek *et al.*, 2002), and would have favourable impact on digestive health of the growing rabbit (Skrivanova and Marounek, 2005). Some fatty acids, such as omega3 class, would also be implicated in the development of immune response (Fortun Lamothe and Boullier, 2004), and Maertens *et al.* (2005) reported a higher post-weaning viability for young fed a diet having a low n-3/n-6 ratio (1.0 vs 4.4). Besides, fat addition to starter diets would increase the energy intake of kits and contribute to maintain a good body nutritional condition. Thus, it would favour a harmonious digestive maturation and immune system development, thus reducing weaning risk and improving resistance to digestive troubles.

3.6. Feed intake regulation and digestive health of the growing rabbit.

Usually, studies on intake regulation aim at analysing the effects on the carcass quality of the growing rabbit, or at analysing the digestive efficiency. But, more recently some studies deal with the relationship between intake level and digestive trouble incidence, including a study with an experimental ERE infection. The effect of a quantitative linear reduction of the feed intake level (100 to 60%) on digestive health and growth of the rabbit was measured through a large-scale study (6 experimental units, 2000 rabbits per treatment, Gidenne *et al.*, 2003). During feed restriction, the mortality and morbidity rates were significantly reduced (resp. from 12 to 3.5% and from 12 to 6% for ad-libitum + 90% feeding level vs 70+60%). The feed restriction during 20d after weaning reduced the growth rate proportionally. Thereafter, returning to an ad-libitum feed intake led to a compensatory growth and to a higher feed efficiency. During the whole fattening period, the live weight loss of the more restricted rabbits (60%) was 7.7%, compared to control rabbits fed ad-libitum since weaning.

Moreover, Boisot *et al.* (2003) also demonstrated a similar positive effect of feed restriction when rabbits were challenged with ERE inoculum. Physiological mechanisms explaining such a favourable effect of reducing the intake level on diarrhoea incidence remain to be studied. Further similar results were also obtained more recently by reducing the intake level through a time restriction for water consumption (Boisot *et al.*, 2004; Verdelhan *et al.*, 2004). Consequently, strategies for controlling the intake of the young after weaning have been rapidly spread for professional breeders, parallel to the development of new automatic feeding and watering equipment.

Conclusion

The interaction between nutrition and digestive pathology has been mainly explored for the growing rabbit, after weaning. The favourable impact of quantity and quality of fibre fractions on digestive health has been demonstrated. The nutritional preparation of the young before weaning is probably a key step determining the digestive health of the growing rabbit (see subchapter 4.4). However, the knowledge of the digestive maturation of the young rabbit requires to be improved. This should provide new concepts for the nutrition of the young between 3 and 5 weeks old.

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4.4. Nutrition of the young and growing rabbit: a comparative approach with the doe

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Introduction

During the first 20 days of life, young rabbits ingest exclusively maternal milk. Later, during weaning period, usually from 21 to 35 days of age, the amount of ingested milk decreases while solid food intake increases. However, since weaning rabbits are fed the same diet of their mothers, the direct effect of the diet on the digestive physiology and performance of young rabbits is hardly distinguishable from its indirect influence through the ingested milk.

The following sections will present the main differences in nutritional requirements of highly productive does and their kits before weaning, also in view of the possibility of feeding them separately. Moreover, part of the chapter will be dedicated to the factors affecting protein retention and nitrogen excretion in growing and reproducing rabbits, in view of reducing environmental pollution from rabbit production.

1. Protein and amino acid requirements

The total protein requirements of rabbits are usually expressed as the digestible protein (DP) to digestible energy (DE) ratio, since dietary DE concentration widely varies in the commercial feeds and because of chemostatic regulation of appetite in rabbits. Under commercial conditions, rabbit does need a minimum of 11.6 g DP/MJ DE ratio, but to maximize fertility, milk production and the survival and growth of suckling rabbits this ratio must be increased until 12.5 g DP/MJ DE (Xiccato, 1996; De Blas and Mateos, 1998; Xiccato and Trocino, 2005). Higher levels (14.3 g DP MJ^{-1} DE) may increase diarrhoea incidence and environmental pollution, decrease feed intake and milk production and impair body condition (Fraga, 1998).

In two consecutive reproductive cycles, García-Palomares *et al.* (2006a) showed that decreasing DP to DE ratio from 13 to 11.5 g/MJ (from 18.4 to 16.1% crude protein, CP) in the late lactation (21 d to 35 d) did not affect performance of rabbit does and their litters. Taking into account also the decrease of milk yield, protein supply to lactating does after the 21th d of lactation might decrease until 11.5 g DP/MJ DE, which corresponds to the lowest value recommended by Xiccato (1996) and de Blas and Mateos (1998) for highly productive does.

The standard CP concentration of the commercial diets for lactating does (18.0-18.5%) also seems to exceed the requirements of young rabbits during the weaning period. In the last two weeks of lactation, milk has high protein concentration (37-40% DM, Fraga *et al.*, 1989; de Blas *et al.*, 1995; Pascual *et al.*, 1999) and high-quality milk protein still accounts for about one third of the total protein intake of weaning rabbits.

Literature on the most limiting amino acids (AA) reports that a minimum level of 0.48 g digestible lysine, 0.37 g total sulphur AA and 0.42 g threonine/MJ DE are required to maximize feed intake of lactating does, while higher levels (0.50, 0.44 and 0.47 g/MJ DE, respectively) are needed to optimize reproductive efficiency in terms of fertility and number of weaned rabbits/cage/year (Maertens and de Groote, 1988; Taboada *et al.*, 1994, 1996; de Blas *et al.*, 1998). On the other hand, an excess of threonine (higher than 0.47 g/MJ DE) impairs feed intake and performance.

To maximize milk production, an increase in lysine supplementation (from 0.44 until 0.56 g

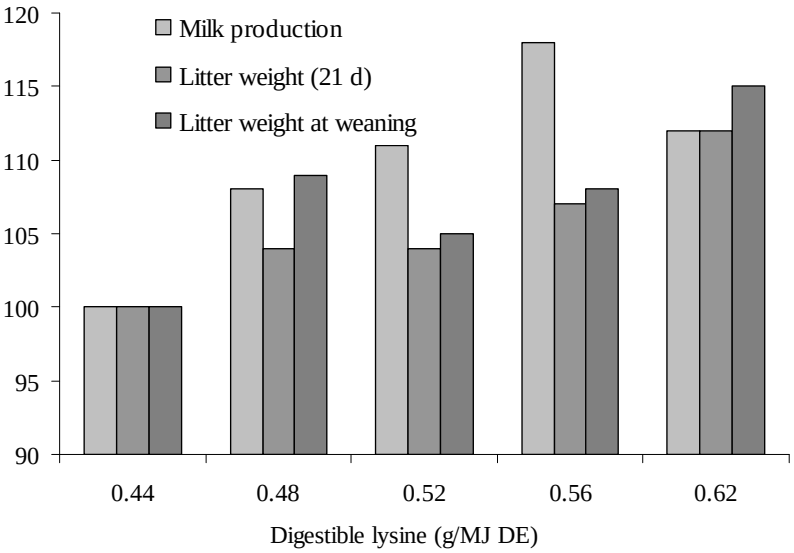

Figure 1. *Effect of digestible lysine concentration of the diet (g/MJ DE) on productive traits (0.44 g/MJ DE = 100) (Taboada et al., 1994).*

digestible lysine/MJ DE) is required (Figure 1). A higher dietary lysine concentration (0.62 g digestible lysine/MJ DE) has a positive effect on growth rate and litter weight at 21 d and at weaning. On this basis, lysine requirements of suckling rabbits might be higher than those of rabbit does, while no difference has been reported for other AA.

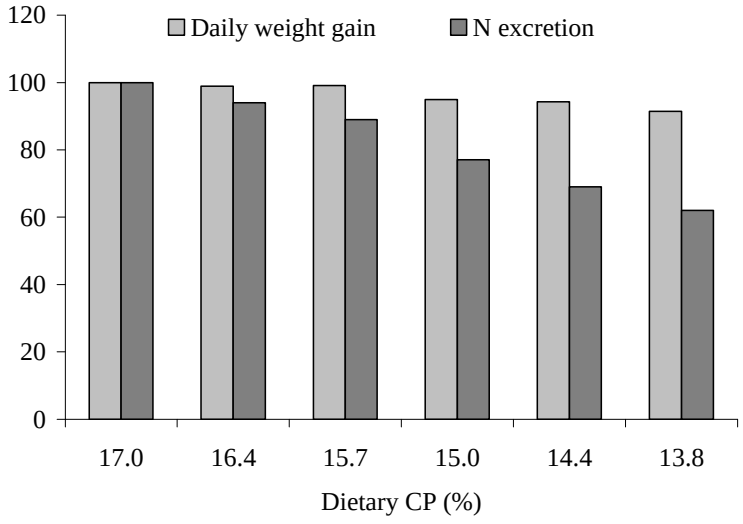

Figure 2. *Daily weight gain and nitrogen excretion in rabbits (32 to 74 days of age) according to dietary CP concentration (17% CP = 100) (Maertens et al., 1997).*

2. Protein retention and nitrogen excretion

The European Directive on the reduction of nitrate in the soil and water (91/676/EC) requires from each member state reference values for N excretion of all livestock as well as the definition of feeding and management strategies to control environmental pollution.

The reference values proposed in Europe (ERM/AB-DLO, 1999) are mainly based on North European conditions. However, large differences exist among Countries especially in the case of rabbits; moreover several feeding and management factors can affect N balance in rabbit farms both in the reproductive and fattening sectors (Maertens *et al.*, 2005; Xiccato *et al.*, 2005).

2.1. Dietary protein level

Nitrogen excretion is strictly dependant on dietary CP level, which usually changes with productive category and, in reproducing does, with physiological state (Xiccato, 1996; de Blas and Mateos, 1998; Xiccato and Trocino, 2005). In commercial conditions, CP concentrations vary from 16.0-17.0% in fattening diets to 16.5-17.5% in weaning diets until 17.5-18.5% in lactation diets (Maertens *et al.*, 2005).

In fattening rabbits, once the limiting amino acids (AA) requirements are satisfied by synthetic AA supplementation, dietary CP may be reduced below 17%, therefore decreasing N excretion without impairing productive performance (Maertens *et al.*, 1997). Only below 13.8% CP, daily weight gain impairs (-9%), but N excretion is reduced by 38% (Figure 2).

According to García-Palomares *et al.* (2006b), decreasing CP concentration from 16 to 14% in diets supplemented with the most limiting AA did not impair growth performance of young rabbits either in the first (35 to 49 d) or in the second phase (49 to 63 d) of fattening. When rabbits are slaughtered later at a higher weight, as in the typical Italian condition, the adoption of feeding programs based on decreasing dietary CP during fattening cycle would permit to satisfy the protein requirements that decrease with age, thus

guaranteeing the highest performance and controlling N excretion at the same time. The best control of N excretion can be realized in the second phase of growth, when N supply can be reduced substantially without impairing productive performance (Maertens *et al.*, 1997; Maertens and Luzi, 1998; Trocino *et al.*, 2000, 2001). In fact, reducing dietary CP from 16 to 14% in the period from 32 to 56 d of age reduced daily growth and body N retention (-6%) and N excretion at a similar extent (-7%) (Trocino *et al.*, 2000). On the contrary, in the second period of growth (56 to 77 d), a reduction of dietary CP from 15.4 to 14.3% decreased N excretion by 9% without any impairment of daily growth and N retention. A further decrease of dietary CP until 13.1% permitted to reduce N excretion by 15% in comparison with the control diet (15.4% CP) while decreasing growth and N retention only by 3%.

As discussed above, protein and AA requirements of reproducing does are largely satisfied by feeding the current lactation diets. Therefore, a reduction of dietary CP during lactation until 17% should not impair either doe reproductive performance or milk yield and litter growth. Taking into account that lactation diet represents about 1/3 of the total feed consumed in a close-cycle farm (reproduction and fattening sectors), advantages in terms of N excretion reduction would be of great importance.

A better control of N excretion could be realized by increasing knowledge on AA requirements and improving the characterization of protein and AA value of raw materials and diets. In fact, some discrepancies in the dietary AA concentration recommended by the different studies could be partially explained by methodological differences in the determination of AA digestibility. As reviewed by Carabaño *et al.* (2000), the use of faecal digestible AA could improve the definition of requirements compared to total AA. However, in rabbits the contribution of ingested soft faeces to the total AA intake (particularly lysine and threonine) is higher than its contribution to CP intake (Nicodemus *et al.*, 1999b). On the other hand, true ileal digestibility has methodological disadvantages and its determination requires surgical cannulation of the rabbits and endogenous losses determinations. Compared to true ileal digestibility, apparent digestible units (ileal or faecal) under-estimate CP and AA digestibility of feeds, due to the importance of the endogenous nitrogen on the ileal and faecal flux, whereas true faecal digestibility over-estimates it. At the present, the use of this latter unit is restricted due to the scarce published information. Requirements expressed as faecal digestible AA are available only for the most limiting AA (lysine, methionine and threonine). Similarly, aa composition and digestibility at faecal and ileal level are known only for few raw materials (Taboada *et al.*, 1994 and 1996; de Blas *et al.*, 1998; García *et al.*, 2005; Llorente *et al.*, 2005b) (Table 1)

.

Table 1. Apparent and true ileal digestibility (%) of different feedstuffs in rabbits (García *et al.*, 2005; Llorente *et al.*, 2005).

	Apparent ileal digestiblity[1]				True ileal digestibility[1]			
	CP	Lys	Met	Thr	CP	Lys	Met	Thr
Sunflower meal	80.7	84.5	93.8	73.8	86.1	91.1	96.7	84.6
Soybean meal	87.7	92.3	91.5	80.3	94.5	96.4	95.2	90.3
Soybean hulls	23.9	55.7	50.1	7.51	46.5	66.6	59.8	36.4
Full fat soybean	82.8	89.6	88.6	74.4	91.5	94.6	93.1	86.5
Lucerne hay	59.1	59.4	74.4	56.2	74.2	71.7	84.2	75.2
Barley grain	61.9	61.6	80.3	45.7	79.6	79.2	89.8	72.5
Wheat bran	52.9	47.2	69.0	43.9	69.8	65.2	79.1	74.4

[1]CP: crude protein; Lys: lysine; Met: methionine; Thr: threonine

2.2. Dietary energy level and DP to DE ratio

High-fibre low-starch diets with low DE concentration have been largely used in the last decade to reduce the risk of digestive disorders, like rabbit epizootic enteropathy (Gidenne, 2003). However, because of the chemiostatic regulation of appetite, when lowering DE concentration, feed intake increases and, if dietary CP concentration remains unchanged, DP to DE ratio and N intake increase. Since growth rate is not modified, N retention remains constant thus increasing N excretion. As an example, when DE concentration decreases from 10.5 to 8.8 MJ/kg and dietary CP

concentration is maintained at 15% with 70% digestibility, DP to DE ratio increases from 10.0 to 12.0 g DP/MJ DE. As shown in Figure 3, body N retention remains unchanged while daily N excretion (faecal + urinary) increases by 20%.

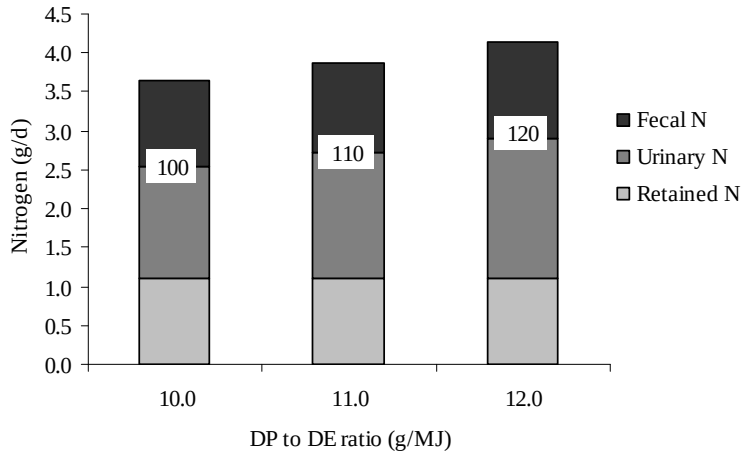

Figure 3. *Daily N retention and excretion (faeces and urine) according to dietary DP to DE ratio.*

2.3. Numerical productivity of rabbit does and slaughter weight

The numerical productivity, i.e. the number of produced rabbit/doe/year, affects directly the amount of excreted N and is in its turn influenced by several factors. The use of more or less intensive reproductive rhythms determines great differences in reproductive efficiency (Maertens *et al.*, 2005). The number of produced rabbits/doe/year can increase from 35-40 in does submitted to extensive rhythms (post-weaning mating) to 45-50 in the case of intensive rhythms (mating 5-12 d post-partum). In a close-cycle farm, with both reproductive and fattening sectors, N excretion can be referred to the reproducing doe including its offspring produced during a year. In this case, excreted N/doe/year depends both on numerical productivity and slaughter weight of fatteners. According to the model proposed by Maertens *et al.* (2005), excreted N increases from 5.24 kg/year in does producing 35 fatteners of 2.25 kg slaughter weight to 9.25 kg/year in does producing 50 fatteners slaughtered at 2.75 kg (Table 2). Similar figures were obtained in a survey on 54 rabbit farms in the Veneto Region (North-East of Italy): in the close-cycle farms, the doe and its offspring (43 rabbits slaughtered at 2.5-2.6 kg/year) ingested on average 11.2 kg N/year and retained 3.8 kg N/year, thus excreting 7.4 kg N/year (Xiccato *et al.*, 2005). If N excretion is expressed on different productive units, e.g. the total number of rabbits produced/year in the farm or the total weight of rabbits produced, excreted N was 0.172 kg per rabbit produced and 0.069 kg per kg produced.

Basing on the above mentioned studies, we can easily estimate total N excreted in the farm based on rabbit slaughter weight, dietary crude protein and the number of reproducing does. With an average CP concentration of feeds consumed in the farm (approximately 1/3 lactation diet, 1/3 weaning diet and 1/3 fattening diet) of 17% and 45 produced

Table 2. Nitrogen excretion (kg/doe/year) according to doe numerical productivity (rabbit produced/doe/year) and fattener slaughter weight (Maertens *et al.*, 2005).

	Rabbit produced/doe/year			
Slaughter weight (kg)	35	40	45	50
2.25	5.24	5.60	5.97	6.37
2.50	6.65	6.93	7.42	7.68
2.75	8.42	8.82	9.08	9.25

Table 3. N excretion (kg/doe/year) according to average dietary protein level and slaughter weight of fattening rabbits (close-cycle farm with 45 produced rabbits/doe/year).

	N excretion (kg/doe/year)	
Slaughter weight (kg)	Diet CP 17%	Diet CP 16%
2.25	6.23	5.67
2.50	7.75	7.08
2.75	9.50	8.71

rabbits/doe/year, N excretion of the doe and its offspring increases from 6.23 to 9.50 depending on the slaughter weight (Table 3). The reduction of average CP level from 17 to 16% permits a decrease of total N excretion by 8-10%.

3. Fat nutrition

Fat addition is commonly used to increase the energy value of rabbit diets with well-known effects on reproductive performance (Xiccato *et al.*, 1995; Xiccato, 1996; Fortun-Lamothe, 1997; Pascual *et al.*, 2003), growth and meat quality (Fernández and Fraga, 1996; Maertens, 1998; Ouhayoun, 1998; Xiccato, 1999; Fernández *et al.*, 2000; Dalle Zotte, 2002). Recent literature reports also its effects on young rabbits around weaning.

Fat digestion and absorption processes in rabbits are similar to those observed in other non-ruminant species (Xiccato, 1998). Gastric lipase permits to hydrolyse the naturally emulsified fat in milk and accounts for most of the lipolytic activity in suckling rabbits (Marounek *et al.*, 1995). When kits begin to assume solid feed, fat digestion occurs in the small intestine since triacylglycerols require emulsification.

The evolution of digestive physiology and fat digestion should support variations in ether extract (EE) digestibility with age. Suckling rabbits are capable of utilising milk fat efficiently, showing a high lipase activity (Dojana *et al.*, 1998; Debray *et al.*, 2003), which, in turn, would permit them to utilise efficiently fat added to starter diets. Parigi Bini *et al.* (1991) assessed separately the digestibility of milk and solid feed during weaning (from 21 to 26 d of age) by multiple regression and found an almost complete digestibility of milk EE (97%), while a lower digestibility of pelleted food EE (74%). Debray *et al.* (2003) found higher apparent EE digestibility (>80%) before 38 d and a significant reduction in fat digestibility from 38 to 52 d, in agreement with the observations of Fernández *et al.* (1994). Other authors, however, found EE digestibility increasing with age (Evans and Jebelian, 1982, Xiccato and Cinetto, 1988). The discrepancies among studies may arise from either the different methods of calculating digestibility (e.g. correction or not for the rapidly increasing intake of solid feed during the first weeks after weaning) or the analytical methods for EE determination in feed and faeces (e.g. acid hydrolysis pre-treatment; extracting solvent) (EGRAN, 2001).

Ether extract digestibility is largely affected by the level and source of dietary fat. In non-added-fat diets, EE digestibility is rather low (45-65%), since lipids are mainly non-triacylglycerols linked to vegetal cell walls (Xiccato, 1998). When animal or vegetal fat is added, EE digestibility substantially increases since added fats are much more easily available and digestible (Maertens *et al.*, 1986, Debray *et al.*, 2003). Also in early weaned rabbits, a significant increase in EE digestibility was found when increasing levels of fat (vegetal or animal) in starter diets (Xiccato *et al.*, 2003a and 2004).

Despite the chemostatic control of appetite, dietary fat addition generally results in higher DE intake accompanied by an only moderate reduction of feed intake, thus improving daily weight gain and feed efficiency (Maertens, 1998; Fernández *et al.*, 2000). Fat addition at high levels (>9%), however, may impair rabbit performance.

The supplementation of lactation diets with fat containing high proportion of n-3 PUFA influences milk fatty acid composition (Pascual *et al.*, 1999) and may have a positive indirect effect on rabbit viability after weaning (Maertens *et al.*, 2005).

Fat addition to starter diets seems increase the energy intake of weaning kits and body fat concentration, favouring at the same time a harmonic digestive physiology and immune system development, thus reducing weaning risk and improving resistance to illness (Fortun Lamothe and Drouet-Viard, 2001). According to Gidenne and Fortun-Lamothe (2002), dietary fat should be higher than 6.0% from 18 to 25 days and higher than 4.0% from 25 to 32 days. Xiccato *et al.* (2003a) found that a starter diet for early-weaned rabbits with 2% animal fat addition (EE 4.1% DM) increased feed efficiency ($P<0.001$) and empty body fat and energy concentration at 32 days of age compared to a standard weaning diet without added fat (EE 2.0% DM) (Table 4).

Similarly, EB fat increased from 5.4 to 6.6% at 42 d of age when increasing EE from 2.8 to 5.8% DM in starter diets by adding vegetal fat (Xiccato *et al.*, 2003b). A high live weight and body fat concentration are usually associated with an improved viability during post-weaning period (Morisse, 1987; Szendro, 2000).

When EE concentration in starter diets was further increased from 5.0 to 6.5% DM, no positive effect on growth performance from 21

Table 4. Empty body (EB) composition in rabbits at 32 days of age (Xiccato *et al.*, 2003a)

	Diet EE (% DM)		Prob.
	2.0	4.1	
EB water (%)	74.7	74.2	<0.01
EB fat (%)	4.1	4.5	<0.01
EB protein (%)	16.6	16.6	NS
EB energy (MJ/kg)	5.71	5.92	<0.01

to 32 days of early weaned rabbits was recorded, while mortality increased (P<0.10) (Xiccato *et al.*, 2004).

4. Starch/fibre ratio

Digestive maturation, and in particular amylase activity, is not fully achieved in suckling rabbits (see 4.1). Since nutritional needs of young rabbits and does differ, several authors (Maertens and De Groote, 1991; Mousset *et al.*, 1993; Gidenne and Fortun Lamothe, 2002) recommend feeding suckling rabbits until weaning separately from their mothers and with a starter diet high in fibre (NDF>35%; ADL>5%) and low in starch (<12%), or to find a feeding strategy with a nutritional compromise among doe and litter (Fortun-Lamothe *et al.*, 2005). However, De Blas *et al.* (1995) observed the best performance both in rabbit does and suckling rabbits given diets containing 19% starch and 32% NDF, in comparison with diets higher or lower in starch. In fact, decreasing starch from 26 to 19% reduced kit mortality during late lactation (21 to 30 d of age); a further decrease of starch to 13%, however, did not reduce kit mortality, but impaired feed intake and daily growth of weaning rabbits. Due to the higher milk production of does fed 19% starch diet, litter weight at weaning and feed efficiency were higher with this intermediate feeding treatment.

Similarly, Debray *et al.* (2002) observed higher feed intake (+13%) and litter weight at weaning (+6%) and lower mortality (0.8% vs 5.2) from 25 to 32 d in young rabbits fed a diet with 17% starch compared to rabbits fed a diet with 14% starch. These results were recently confirmed by Nicodemus *et al.* (2005) who observed, in the period from 21 until 25 d, higher feed intake (+39%) in suckling rabbits fed a diet with 19% starch compared to 10% starch along the first four lactations of rabbit does. Conversely, milk intake during lactation tended to be 11% lower (P<0.10) in kits fed with the 19% starch diet and therefore the average daily weight gain in the whole lactation was not affected by treatments. No negative effect of dietary starch level on kit mortality was observed during lactation. This effect was also confirmed after weaning (see subchapter 4.3).

When comparing two diets with different NDF and starch levels (30 and 20% vs 25 and 30%, respectively), litter feed intake from 21 to 25 d was higher with the low-fibre high-starch diet (115 vs 60 g/d), although litter growth rate was not affected due to the lower milk intake of the same kits (Nicodemus *et al.*, 2003a). According to the above mentioned results and the positive data obtained by Messager (1993), feeding young rabbits with highly digestible pre-starter diet, young rabbits seem to perform better when receiving a low-fibre diet during the weaning period.

Dietary levels of 30% NDF and 20% starch have been proposed as optimal to maximize daily weight gain and feed efficiency and to minimize mortality of weaned rabbits from 25 (weaning) to 39 d of age (Gutiérrez *et al.*, 2002; Nicodemus *et al.*, 2003b and 2004). Fortun-Lamothe *et al.* (2005) proposed a diet with a similar level of NDF (30.6%) and lower starch (9.5%) replaced by fat (5.5%) as a compromise between doe and young nutrition in comparison with a diet lower in NDF (27.6%) and higher in starch (19.0%): the results of a large scale study (6 sites and about 550 does and 9000 rabbits) showed that the high-fat low-starch diet reduced the mortality of young rabbits without impairing their growth or the reproductive performances of does.

5. Type of fibre and particle size

The dietary concentration of crude fibre (CF) is not sufficient to adequately define the fibre requirements of growing rabbits to prevent digestive disorders (Gidenne, 2003), since chemical composition and physical structure of plant cell walls vary widely among fibre sources (Maertens *et al.*, 2002).

The dimension of fibrous particles is also important due to the influence on transit time and likely on digestive efficiency and caecal fermentation activity (García *et al.*, 1999). Recently, Nicodemus *et al.* (2006) reported that a high proportion (>20%) of large particles (>0.315 mm) improved productive traits of weaning rabbits and lactating does. Below 20% large particles, doe feed intake and milk production decreased as well as litter feed intake and weight. The effect of particle size reduction on feed intake and performance was found more negative in weaning than in fattening rabbits.

The degree of lignification of the cell wall has been reported as one of the main factors influencing caecal retention time (Gidenne *et al.*, 2001). A minimum ADL concentration of 5.9% DM (in diet with NDF 39% DM) is required to maximise feed intake of lactating does, milk production and litter weight (Nicodemus *et al.*, 1999a). The effect of dietary ADL on litter weight at weaning was found to be less significant, since no differences in feed intake and growth rate of suckling rabbits were found between 21 and 30 days of age. ADL requirements for maximum performance appeared higher in lactating does than in weaning rabbits, which might be related to the higher energy requirements and feed intake per unit of body weight in does compared to kits, as ADL has a positive effect on rate of passage.

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4.5. Strategies for doe's corporal condition improvement – relationship with litter viability and career length

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1. Introduction

Rabbit meat production has undergone great changes in the last two decades. We have moved on from more or less traditional production systems to other, more intensive ones, which have made the adjustment of the nutritional needs of the animals necessary in line with the new demands (Maertens, 1992). The nutritional needs of kits during the fattening phase have changed as a result of selection by growth rate - between +0.45 and 1.23 g per day and generation of selection - however, it is the reproductive does that have suffered the effects of these improvements and the new production systems to a greater extent.

Genetic selection programmes in reproductive rabbit does have mainly focused on improving litter size, either at partum or weaning. These programmes produce an effective increase of litter size between 0.05 and 0.13 live-born kits per generation of selection (Rochambeau *et al.*, 1994; Gómez *et al.*, 1996). This selection criterion, along with artificial insemination (AI) of the does with semen from bucks selected for growth rate, has clearly increased the litter's demand for milk.

On the other hand, the intensification of the reproductive rhythms gives rise to competition between the mammary glands and foetuses, which is usually detrimental to foetal growth if the needs are not well covered (Fortun and Lebas, 1994b). The needs of reproductive rabbit does may therefore have increased considerably in recent years. For this reason, a great number of research works has centred on the determination of the nutritional needs of reproductive does and the appropriate nutritional strategies for feeding management. In this respect, very detailed revisions may be found in the bibliography where recommendations are made for the determination of the nutritional needs in reproductive does at different points in their reproductive cycle (Parigi-Bini and Xiccato, 1998), or on nutritional strategies for a better control of the does (Fortun-Lamothe, 2006). However, most of these works aim at determining nutritional strategies that maximise the productivity of the does (ingestion, milk production...) at certain stages, often without considering the long-term effects (body condition, health status, ...).

In the current productive context, productivity criteria are being sidelined by others that take more into account the welfare of the animals and the general health status of the farm. However, these criteria must not hinder the competitiveness of the farm. The search for long-term strategies that uphold these criteria (general farm health, longevity, body condition) would take into account the possible collateral effects resulting from isolated strategies (Pascual, 2005). A suitable strategy for the feeding of reproductive does would therefore have to consider short-term productive criteria as well as long-term ones (for example body condition, life span and health status of the doe).

In the present chapter, the efforts made in this respect in recent years are reviewed, analysing the long-term nutritional needs and feeding strategies for reproductive does.

2. New advances for body condition evaluation

2.1. In vivo methods

The slaughtering of animals followed by carcass dissection and/or chemical analysis has been frequently used in rabbit species. It provides precise and cost-effective means to obtain the evaluation of the body condition of an animal. Using this method, Parigi-Bini and Xiccato (1998) determined the coefficients of energy utilisation for maintenance, foetal growth and milk production which could be used to calculate energy balance in reproductive does. However, this destructive method doesn't allow sequential studies of the same subject over several reproductive cycles. It is the reason why much effort has been made recently to find non-destructive methods to predict the body composition (Szabo *et al.*, 1999). Several methods validated for other species have been tested in the rabbit. Some, such as infrared radiation (NIRS, Masoero *et al.*, 1992) or deuterium oxide dilution (Fekete, 1992) must be rejected because they are unreliable in this species. X-ray tomography (Romvari *et al.*, 1996) or imaging by nuclear magnetic resonance (MRI; Kover *et al.*, 1998) were found to be useful but very expensive and require anaesthesia of animals which, could induce physiological disturbance and modifications of the later reproductive performance.

Since the perirenal adipose tissue is the most important location of lipids storage in rabbits, several research teams tested the use of ultrasound to assess the body composition of rabbit does. The animals are immobilised in a box and the scanning area is shaved. The thickness of perirenal fat is measured at a fixed anatomical reference point: 8^{th}-9^{th} thoracic vertebrae (Pascual *et al.*, 2000b) or 3 cm ahead the 2^{nd}-3^{rd} lumbar vertebrae (Dal Bosco *et al.*, 2003). Pascual *et al.* (2000b) demonstrated that this technique is reliable in young rabbit does ($R^2 = 0.90$; CV = 5.1%). Subsequently, Pascual *et al.* (2004) tested the accuracy of this method to estimate the body composition of does at different physiological status (parturition, lactation, weaning and pregnancy). Their results showed that it is necessary to use specific equation according to the physiological status of reproductive does ($R^2 = 0.67$-0.76; CV = 7.6-9.8 %). The technique is not accurate to estimate the body composition of does at the end of gestation, probably due to the moving of adipose tissue inside the abdominal cavity when the foetuses develop. This technique was used successfully to study the influence of nutrition on the evolution of female's body condition (Pascual *et al.*, 2002a; Quevedo *et al.*, 2005).

The TOBEC method (Total Body Electrical Conductivity) is based on a measurement of electrical conductivity of the animal using a detection chamber. The method requires no specific preparation and could be performed on steady state animals. Each doe passes at least three times through the detection chamber. The TOBEC value (E-value) is positively related with the water content and negatively related with the lipids content of the subject. The method has the disadvantage of not providing information about the anatomic distribution of adipose tissues. Additionally, it can be affected by variations in the gut contents, and standardisation of the measurement conditions is therefore necessary (delay from meal). TOBEC method was recently validated in reproductive does by Fortun-Lamothe *et al.* (2002) using a combination of E-value and the animal's live weight. It appears to be accurate in predicting water and energy content in reproducing rabbits with high R^2 (≥ 0.84) and low CV (≤ 4 and $\leq 12\%$, respectively). The high variation (CV = 24%) for prediction of the body lipid content limits the reliability of the method for this variable despite a high correlation coefficient ($R^2 \geq 0.82$). The method was used to study the relationship between fertility and body condition (Bolet and Fortun-Lamothe, 2002), and the effects of feeding strategy around weaning on evolution of body condition (Fortun-Lamothe *et al.*, 2005).

Recently, Bonnano *et al.* (2005) tested the possibility of using body condition scoring to evaluate the nutritional status of lactating does. This method is widely used for several livestock animals (cows, ewes, sows), being much more accurate than body weighing or a simple eye appraisal. However, until now it was not used in rabbit probably because the main reserve of body is located inside the abdominal cavity as perirenal fat, and is not accessible by simple palpation. The authors proposed a manual evaluation of some indicators of the body state. Bone protrusions (spinous process) and fullness of muscle are evaluated both in loin and rump regions and muscle thickness is evaluated on hind leg. These traits were subjectively scored using 0, 1 and 2 for poor, intermediate and good condition, respectively. The hind leg score was weakly correlated to body traits but highly correlated to does parity ($r=0.14$; $P=0.002$). The score (0-2) of the loin and rump regions were added to obtain the body condition score (BCS, 5 classes from 0 to 4). BCS was highly correlated with total body fat ($R^2 = 0.74$, $P<0.001$) but not with the relative weight of hind leg ($R^2 = 0.13$, NS). An interesting relation was found between BCS and fertility. Therefore, furthers studies are necessary to better determine the reliability of this method which present the advantage of being very simple.

2.2. Blood indicators of the nutritional status

Several blood metabolites, including glucose and non esterified fatty acids (NEFA), or hormones,

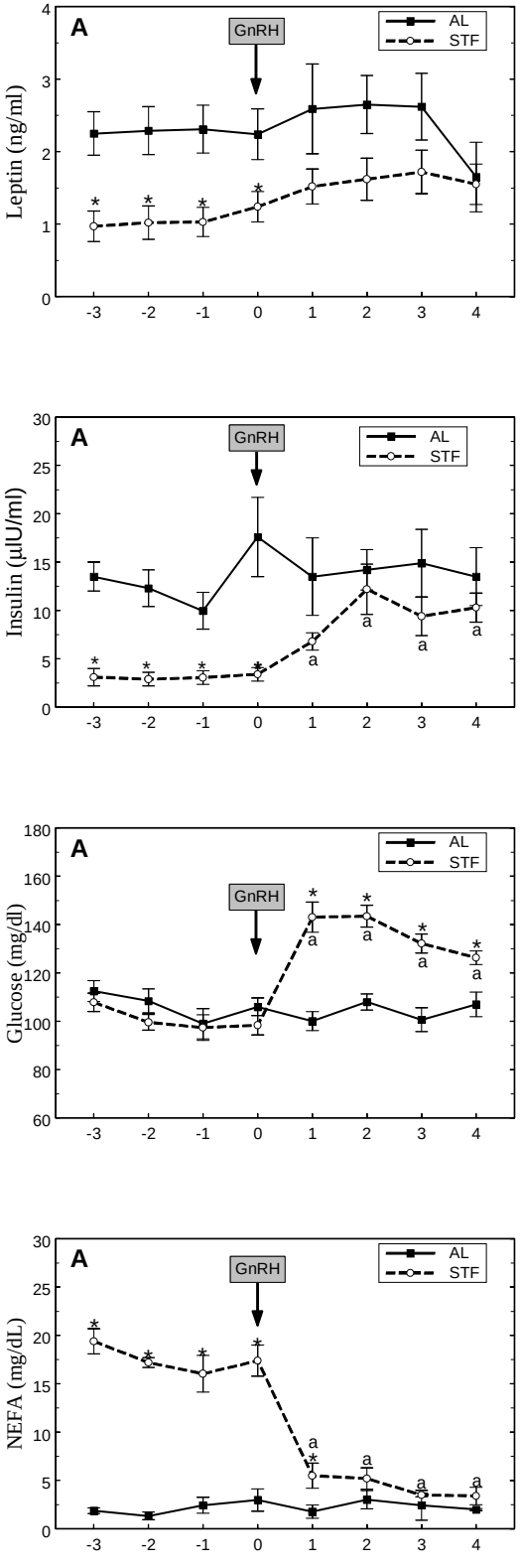

Figure 1. Plasma leptin, insulin, glucose and non esterified fatty acids (NEFA) concentrations in does fed *ad libitum* (AL) or fasted for 24 h (STF), before and after resumption of feeding in fasted rabbits (time 0).

*: significant (P≤0.01) different means between different experimental groups. Letters mark significant differences (P≤0.01) within the same experimental group. (adapted from Brecchia *et al.*, 2005).

nutritional status and/or body condition of animals and could be used as indicators of these parameters.

Glucose may be a good indicator of the energy balance of animals (Chilliard *et al.*, 1998). Long-term underfeeding was found to reduce glucose concentration in rabbits (Rommers *et al.*, 2004), but blood concentration of glucose rises immediately after re-feeding in does fasted from 24-48h (Brecchia *et al.*, 2005; Figure 1). Several authors have observed a fall in glycaemia during the course of gestation in response to the gradual increase in the requirements for foetal growth (Gilbert *et al.*, 1984; Parigi-Bini, 1988). The blood glucose levels are lower in rabbits which are simultaneously gestating and lactating (Fortun, 1994).

Non-esterified free fatty acids (NEFAs) are typically released into the blood stream when glucose levels fall and, as a result, the reduced insulin to glucagon ratio leads to activation of the hormone sensitive lipase which hydrolyses triglycerides to free fatty acids and glycerol. Therefore, NEFA, deriving from the mobilisation of body reserves in the blood, could be an appropriate indicator of energy balance. However, a high variability was observed between individuals in the same physiological status (Xiccato *et al.*, 2005). Recently, Theilgaard *et al.* (2005a) has tested the increase of NEFA blood concentration after adrenergic (isoprotenerol) challenge as a possible tool to evaluate the lipolytic potential in the rabbit does' fatty reserves. NEFA concentration is higher in feed restricted rabbits than in *ad libitum* ones (Figure 1), and the longer the fasting the higher the NEFA concentrations (Brecchia *et al.*, 2005). Circulating levels of non-esterified fatty acids are higher in pregnant rabbits if they are simultaneously lactating, at the start and in the middle of lactation (Fortun, 1994).

Insulin, a peptidic hormone, is released from the pancreas in response to rising glucose levels. Insulin stimulates the formation of glycogen, increases the uptake of glucose by most tissue, inhibits lipolysis and gluconeogenesis and stimulates lipid accretion. Therefore, high insulin levels in blood generally indicate positive energy balance and repletion of fat stores. Fasting leads to reduction in insulin blood levels in growing rabbits (Rommers *et al.*, 2004) and in adult reproductive does (Brecchia *et al.*, 2005 ; Figure 1). Insulin level increases markedly at the beginning of gestation, to stimulate the storage of energy in form of fats, and returns to a basal level at the end of gestation, probably in response to the high nutritional needs of growing foetuses (Fortun, 1994).

Leptin may act as a critical link between adipose tissue and the neuroendocrine axis, indicating the level of energy reserves (Moschos *et al.*, 2002). The plasma concentration of leptin is correlated with the proportion of body lipids and the index of body mass (Considine *et al.*, 1997; Chilliard *et al.*, 1999). In rabbits, Xiccato *et al.* (2005) observed that leptin

levels were higher in females submitted to extensive reproductive rhythm (insemination 26 days after kindling) compared to intensive or semi-intensive ones (insemination 2 or 11 days after kindling, respectively). Brecchia *et al.* (2005) showed that blood leptin levels are lower after 24h-fasting and gradually increased after re-feeding to be similar to those of *ad libitum* does 4 h after re-feeding, suggesting that leptin also signals the current availability of metabolic fuel (Williams *et al.*, 2002).

3. Evolution of doe's body condition

The body composition of rabbit does changes dramatically during their reproductive carrier. An intense body energy deficit has been proved during the first and second lactations, especially in highly-productive hybrid lines whose voluntary feed intake is often unable to cover nutrient requirements for lactation and concurrent pregnancy (Partridge *et al.*, 1986a and 1986b; Maertens, 1992; Xiccato, 1996), which could determine productivity, life expectancy and health status of the female.

3.1. Regulation of voluntary feed intake

The appetite regulation in reproducing rabbits is mostly controlled by chemostatic mechanism, for which the total quantity of energy ingested daily tends to be constant (Pascual, 2004). Young reproducing females ingest a decreasing amount of DE, from 950 to 700 kJ/day/kg $LW^{0.75}$, in the period from weaning to first insemination. During the first pregnancy, daily energy intake further decreases from 600-650 kJ/day/kg $LW^{0.75}$ of DE in the first 25 days until 400-450 kJ/day/kg $LW^{0.75}$ in the last 5 days, due to the increasing volume of foetuses in the abdomen. Energy consumption is higher in lactating females, which can ingest till 1,500-1,800 kJ DE/day/kg $LW^{0.75}$ at the lactation peak and 1,100-1,300 kJ DE/day/kg $LW^{0.75}$ on average. The highest values are recorded with multiparous does (Parigi Bini and Xiccato, 1998). After litter weaning, the does quickly decrease their feed intake in a week at about 35-45% of the lactation level, i.e. 500-600 kJ DE/day/kg $LW^{0.75}$ (Xiccato *et al.*, 2004b and 2005).

3.2. Body condition during pregnancy

Primiparous does undergo wide variations in body composition tissue deposition and energy retention during pregnancy (Parigi Bini *et al.*, 1990, 1991; Milisitis *et al.*, 1996, 1999; Xiccato *et al.*, 1999; Pascual *et al.*, 2002b). During early and mid gestation (0-21 days) feed intake is clearly superior to the needs, so live weight (LW, Parigi-Bini and Xiccato, 1993) and perirenal fat thickness (PFT, Quevedo *et al.*, 2005) increase like in non pregnant does. During late pregnancy (21-30 days), empty body weight (LW-gut content) decreases because of protein and fat losses and transfer of energy to the rapidly growing foetuses. Pascual *et al.* (2002a) observed a reduction of 3.8 mm in PFT during the final 3 days of gestation.

3.3. Body condition during lactation

The energy output in the milk during lactation is exceptionally high in rabbits, compared to other species, due to the intense milk production (200-300 g/day) and its high dry matter (30-35%), protein (10-15%) and fat concentration (12-15%) (Lebas, 1971; Fraga *et al.*, 1989; Parigi Bini *et al.*, 1992; Pascual *et al.*, 1999b). A 4-kg doe producing 250 g/day of milk excretes 752 kJ /day/kg $LW^{0.75}$.

Several works indicate that primiparous does show a clear negative energy deficit during whole lactation perhaps related to their relatively low ingestion capacity compared to their productive level, and/or the relatively good body condition in which primiparous does afford their first lactation. Pascual *et al.* (2002a) observed that primiparous does lost 0.2 mm of PFT during the 3 first weeks of lactation, but PFT losses reached 0.9 mm during the final week. Similar results were obtained by Bolet and Fortun-Lamothe (2002), who

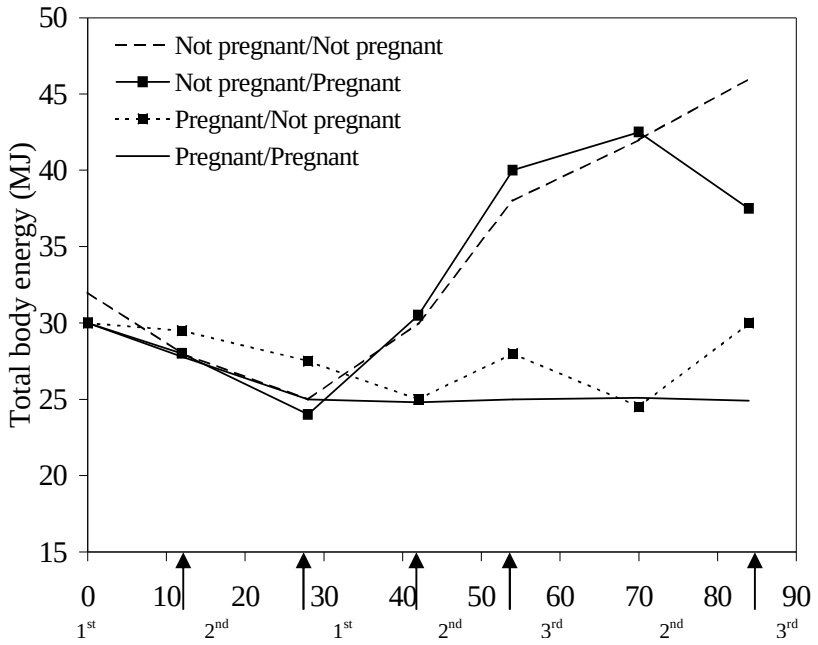

Figure 2. *Evolution of total body energy concentration (TOBEC measurements) in rabbit does from 1st to 3rd kindling and according to their physiological state (Bolet and Fortun-Lamothe, 2002).*

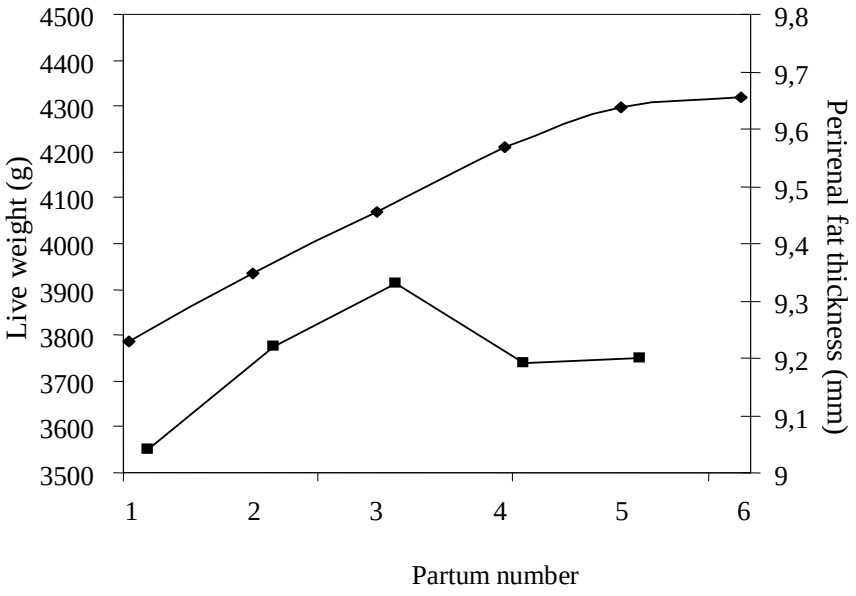

Figure 3. *Evolution of live weight at parturition (♦) and perirenal fat thickness on 11th day of lactation (■) of the does throughout 6 reproductive cycles (adapted from Quevedo, 2005).*

recorded a reduction of 5 MJ in the total energy content estimated by TOBEC in primiparous does, both between days 1 and 11, and between days 11 and 28 of lactation (Figure .2).

However, the energy balance during lactation seems to be different in the case of multiparous does. Unlike the primiparous does, during the first 11 days of lactation an increase in the body reserves of the does of +0.3 mm PFT (Quevedo, 2005) was observed, or of +6 MJ estimated by TOBEC (Bolet and Fortun-Lamothe, 2002). As this point, Quevedo (2005) observed no important modifications of PFT until weaning, when does have a semi-intensive reproduction rhythm (56 day interval between parturitions), whereas Bolet and Fortun-Lamothe (2002) observed a variable behaviour (Figure 2).

On the other hand, Xiccato *et al.* (2004a) observed that multiparous does simultaneously in lactation and gestation (42 day interval between parturitions) maintain an energy deficit during lactation until the third reproductive cycle (-8.36, -3.30 and -2.60 MJ during the 1st, 2nd and 3rd lactation, respectively). Slight difference between authors may be ascribed to the rabbit strain (commercial hybrids or selected pure breeds) and/or the body balance measurement method (comparative slaughter, ultrasound technique, total body electric conductivity).

3.4. Long term evolution

When adapting the body condition of does in the long term is considered, reference to attempts to improve the longevity of our animals is inevitably made, since the life span of a female on a commercial farm mainly depends on its health and body condition, as long as the productive level is optimal. Recently, Theilgaard *et al.* (2005b) analysed, by means of a survival test, the risk of elimination of a reproductive doe based on its body fat status on the 11th day of lactation, showing that elimination risk was significantly higher ($P<0.05$) for those animals with a lower adipose status. This agrees with the hypothesis that the animals need a minimum amount of fat to ensure the resources demanded by suckling kits, without having other functional cost such as, a decrease of longevity.

There are few works that have monitored the body condition of does in the long term. Most authors (Pascual *et al.*, 1998; Fernández-Carmona *et al.*, 2003; Quevedo, 2005) indicate that the does do not reach their definitive adult weight until the fourth or fifth reproductive cycle (Figure 3), and Quevedo (2005) observed that does reached their maximum PFT during the 3rd reproductive cycle, being more or less maintained as of this point, although the evolution is very variable and mainly depends on their reproductive history (Fortun-Lamothe, 2006).

Bolet and Fortun-Lamothe (2002) indicated that the principal factor conditioning the body condition at the third parturition is the fertility, and therefore the simultaneity of lactation with gestation, in the two preceding cycles. In fact, it was observed that the total amount of body energy at the third parturition of the does covered outside lactation is almost double (47 MJ) compared to those covered in both cycles during lactation (25 MJ). Given all of the above, it is necessary to devote a greater research effort in future to determining the most suitable reproductive and nutritional management so that the doe present an optimal body condition, allowing them to confront the different reproductive cycles successfully, but without their life expectancy (longevity on farm) and their health being jeopardised.

4. Strategies for doe's corporal condition improvement

The doe energy deficit may be controlled by means of increased voluntary feed and energy intake, reduced body energy output by anticipating weaning age, or increased body energy recovery by prolonging the kindling-to-kindling interval.

4.1. Effect of nutrition

4.1.1. Feeding the young does

In the first period of growth (until 10-12 weeks), the growth models developed for meat rabbits (Parigi Bini and Xiccato, 1998) can be used. The voluntary DEI of young rabbits kept for reproduction is 900 kJ/day/kg $LW^{0.75}$ on average, and the rabbits fed *ad libitum* reach about 2.4 kg at 11 weeks of age. In the second period (until first mating, 17-18 weeks), the somatic and physiologic development of female advances slowly. The body protein and ash concentrations tend to remain stable at 20% and 3% respectively, while the level of fat depends on age at first mating and feeding regime.

Females are usually mated the first time at 16-18 weeks of age, or at 75-80% of adult weight (Parigi Bini, 1988; Maertens, 1992). From 10-12 weeks to first mating, voluntary DEI slightly decreases from 800 to 700 kJ/day/kg $LW^{0.75}$ and daily weight gain decreases from 35 to 20 g/d (Xiccato *et al.*, 1999). At 17 weeks of age, breeding rabbits given *ad libitum* access to a diet containing 10 MJ/kg DE may reach about 3.4 kg LW and about 18% body fat concentration, which could be excessive if a further fattening occurs during pregnancy (especially in case of pregnancy failure).

To avoid overfattening, feed is often given at a restricted level, but it has been criticised for the likely reduction of feed intake in the successive phases with the accentuation of the risk of a negative energy balance between reproductive cycles (Parigi Bini *et al.*, 1991; Maertens, 1992; Fortun-Lamothe, 2006). A feeding restriction (80-90%) in the first growth period (5-11 weeks), followed by unrestricted feeding, should result in an optimal body condition at 17-18 weeks of age and a voluntary feed intake higher than traditional restricted programs. Feed restriction can continue also in the first part of pregnancy, especially when LW exceeds target weight, while *ad libitum* feeding with a lactation diet could be recommended during the last 2 weeks of pregnancy because of increasing pregnancy requirements (Quevedo *et al.*, 2005). If an *ad libitum* feeding regime is adopted, a growing diet containing a moderate energy level (9.5-10 MJ/kg), rich in fibre and well provided with protein, should be given throughout most of the growing period until the first mating, to allow doe good somatic development and increase voluntary feed intake during subsequent reproductive career (Xiccato, 1996).

To stimulate voluntary feed intake of young does during subsequent reproductive activity, high fibre-low energy diets were given to young females before the first mating (Nizza *et al.*, 1997; Xiccato *et al.*, 1999; Pascual *et al.*, 2002a). Reproductive performance at birth was not affected, while the higher feed intake during lactation and milk production increased litter size and weight at weaning (Pascual *et al.*, 2002a), while body fat and energy deficit during lactation was decreased (Xiccato *et al.*, 1999).

4.1.2. Feeding reproducing does

High energy diets are usually designed on the basis of lactation needs, but due to management issues the does usually also receive the feed outside lactation. During gestation, the energy supplement of the feeds usually produces a reduction in the ingestion of the does due to the energy regulation of the ingestion, but in some cases the females are not able to regulate their ingestion, especially with feeds rich in starch (Pascual *et al.*, 1998; 1999a; 2002b), which gives rise to excessive energetic ingestion. Several works carried out on the long term showed a diminution of the number of live born as a result of a greater mortality rate at partum of those kits whose mothers received feed rich in fat (Partridge *et al.*, 1986a; Parigi-Bini *et al.* (1996) or starch (Pascual *et al.*, 1999). Quevedo (2005) observed a higher ingestion

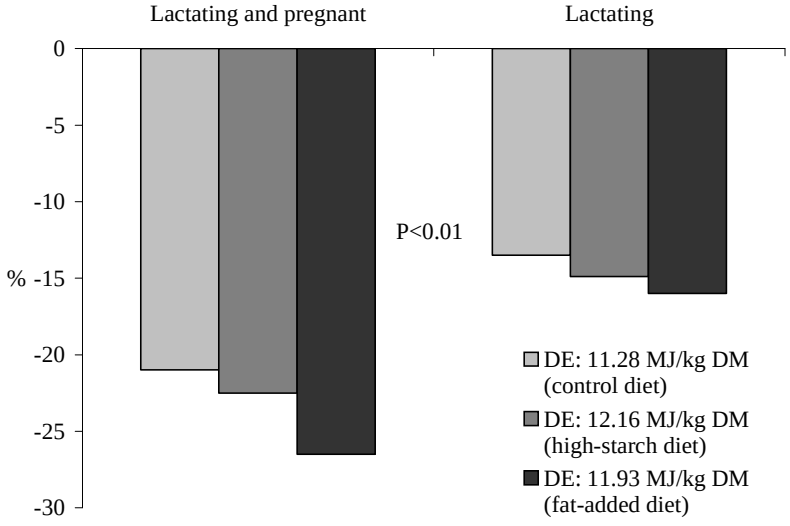

Figure 4. *Body energy balance in empty body of primiparous does at different physiological state given diets with increasing DE concentration (Xiccato et al., 1995).*

of energy and a reduction in litter size at partum of the does that received a high energy diet during gestation. However, these results were not due to the primiparous nor multiparous does whose gestation overlapped with the preceding lactation, but to the multiparous does that did not overlap lactation and gestation. These results suggest that the long-term use of energy-rich feeds must be applied with care outside of lactation.

During lactation, feeding high energy diets increases DEI (Pascual *et al.*, 2003). This effect is even accentuated when fat-added diets are used in comparison with high-starch diets both in primiparous and multiparous does (Xiccato *et al.*, 1995; Fortun-Lamothe and Lebas, 1996; Parigi Bini *et al.*, 1996; Pascual *et al.*, 1998; 2002b). However, a higher dietary energy supply determines an increase of milk production, limiting its beneficial effect on body condition both in primiparous (Xiccato *et al.*, 1995; Fortun-Lamothe and Lebas, 1996) and multiparous does (Pascual *et al.*, 2000), regardless of the energy source (Figure 4).

4.2. Effect of management

4.2.1. Reproductive rhythm

The most diffuse remating program remains the semi-intensive rhythm, which is 11-12 d post-partum, a compromise between the doe need of recovering energy between one reproductive cycle and the next, and the economic demand of increasing the number of kits weaned per year. Post partum insemination implies an excessive exploitation of the doe, which finally results in a strong reduction of reproductive performances and career length. Extensive rhythms imply a too low number of kindling per year and can cause doe overfattening and subsequent impairment of reproductive performance.

Milk production is largely affected by the reproductive rhythms and the different overlapping periods of pregnancy and lactation. Does submitted to intensive reproductive rhythms begin showing decreased milk production after 15-17 d of lactation and evidence a sharp decrease in the last week of pregnancy (Lebas, 1972; Partridge *et al.*, 1986b; Fraga *et al.*, 1989). In fact, nutritional requirements increase consistently after the third decade of pregnancy due to exponential foetal development (Parigi Bini and Xiccato, 1998) and the hormonal changes caused by the imminent kindling that contrast with milk production (Fortun-Lamothe *et al.*, 1999).

One of the main reasons for lengthening the interval between kindling is prolonging the dry period in order to permit complete body energy recovery (Partridge *et al.*, 1984; Cervera *et al.*, 1993). In primiparous does, a severe body energy deficit was observed within the first and second kindling with insemination 12 d post partum (-26% of initial body energy content) but a less serious deficit (-15%) with insemination 28 d post partum (Parigi Bini *et al.*, 1996) (Figure 5a). When multiparous does were submitted to early weaning (21 or 25 d), body energy deficit disappeared in those submitted to semi-intensive (insemination 12 d post partum) and extensive (26 d post partum) rhythms whereas it was severe only in rabbits submitted to the intensive reproductive rhythm (2 d post partum) (Xiccato *et al.*, 2005) (Figure 5b).

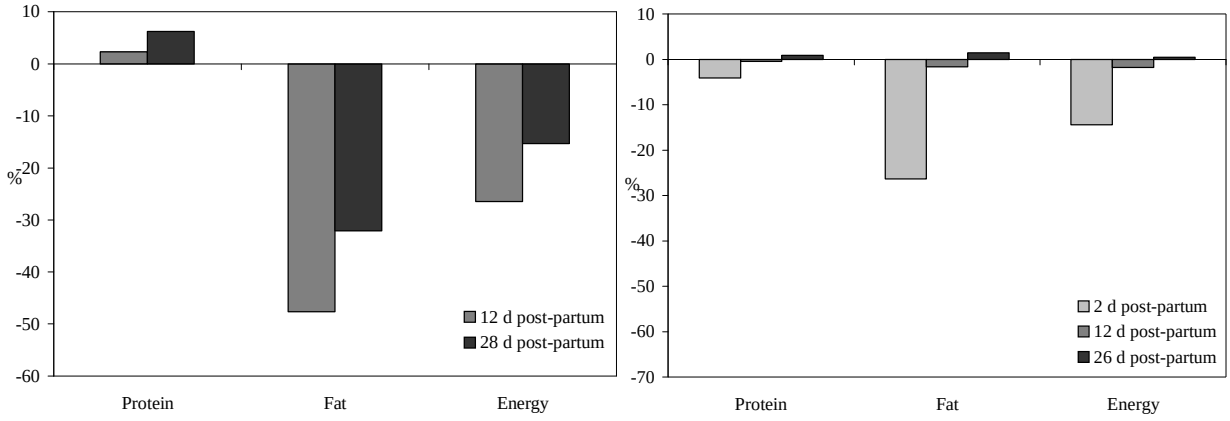

Figure 5. *Effect of reproductive rhythm on body protein, fat and energy balance at kindling in reproducing does: (a) primiparous does with litter weaning at 28 d (Parigi Bini et al., 1996); (b) multiparous does, average of litter weaning at 21 and 25 d (Xiccato et al., 2005).*

Higher reproductive efficiency has been reported in does submitted to extensive rhythms (Parigi Bini *et al.*, 1989; Cervera *et al.*, 1993; Xiccato *et al.*, 2005), even if higher embryonic mortality was observed (Parigi Bini *et al.*, 1996). The role of high prolactine and low progesterone levels in lactating does in reducing the foetal survival rate has been elucidated, while the effect of the nutritional deficit caused by concurrent lactation is less clear (Fortun-Lamothe *et al.*, 1999). An

alternating re-mating rhythm (alternatively 1 d post partum and post weaning associated with weaning at 26 d) may improve both receptivity and fertility with greater respect for doe physiology when compared to a fixed re-mating rhythm (11 d post partum) (Castellini *et al.*, 2003).

4.2.2. Litter weaning age.

Previous research reported a negative correlation between weaning weight and post-weaning mortality (Morisse, 1987; Lebas, 1993) and induced breeders to increase weight by delaying weaning age. On the other hand, more recent studies demonstrated the possibility of successfully anticipating kit weaning age (De Blas *et al.*, 1999; Xiccato *et al.*, 2000; Pascual, 2001).

The major interest in early weaning technique lies in the possibility of reducing the doe body energy deficit, by shortening the lactation length and prolonging the dry period. Milk production requires also a great energy effort in its final period, because milk dry matter and lipid concentrations increase from the 20th day onwards when production begins to fall (Lebas, 1971 and 1972; Pascual *et al.*, 1999b). Moreover, the natural progressive weaning does not permit the complete recovery of the body energy lost during lactation because of the very short dry period.

In does at their first, second and third parturition, reducing weaning age from 32 to 21 d of age improved body energy balance (from –19% to –8% of the initial energy concentration) but was unable to achieve equilibrium (Xiccato *et al.*, 2004a). Also in multiparous does, weaning at 25 d did not prevent body energy deficit (–8% of the initial energy content) while weaning at 21 d only provided a balance that approached equilibrium (-3%) (Xiccato *et al.*, 2005). Early weaning failed to definitively balance the energy deficit in these does because of the substantial decrease in feed intake after weaning (about 40-50% of lactation period). With natural weaning, in fact, feed intake remains at the highest levels also during the last decade of lactation, thus partially permitting the recovery of the body energy lost during the first 20 days. The sudden decrease in feed intake that occurs after early weaning reduces the daily energy surplus and delays the complete restoration of body reserves (Xiccato *et al.*, 2004b, 2005).

Scientific literature on the reproductive performance of does submitted to early weaning is scarce. A negative effect of early weaning on rabbit reproductive performance was also recorded in terms of the lower number of kits born and kits born alive per litter in multiparous does whose litters were weaned at 21 days of age (Xiccato *et al.*, 2004 and 2005). This result could be explained by the effects of weaning on metabolic and hormonal patterns which occurred at the time of foetus implantation from 7 to 11 days of pregnancy (Brecchia *et al.*, 2005; Fortun-Lamothe, 2006). The occurrence of mastitis may also be observed in the field as a consequence of the abrupt interruption of lactation at 21-25 days. When early weaning (25 d) was associated with an early mating (4 d post partum), higher prolificacy and litter size at weaning were observed in comparison with does subjected to a traditional system mating-weaning system (mating 11 days post partum and weaning at 35 days of age) (Nicodemus *et al.*, 2002). These results cannot be clearly ascribed to weaning age or reproductive rhythm, however.

4.3. Effect of genetic selection

In recent decades, reproductive does have been selected for prolificacy criteria with a certain degree of success (García and Baselga, 2002a and b; Tudela *et al.*, 2003), and this reproductive improvement should also have altered their nutritional needs. However, there is little information on the possible effect of genetic selection by litter size on the development of the does, and on the appropriate nutritional strategies for this type of animals based on their genetic level.

The recent development of embryo freezing and transference techniques (García-Ximénez *et al.*, 1996) may provide the opportunity to compare and study the development of live animals of older generations with the current generations. Using this type of techniques, some works in recent years have attempted to find suitable nutritional strategies at the level of genetic selection of the does. Quevedo (2005) and Quevedo *et al.* (2005) recently studied the effect of the selection by litter size at weaning on the development and physiological and productive characteristics of the does, by means of the contemporary comparison of does crossed with 12 generations of differential selection. The does resulting from the crossing of the current generations presented a greater number of live-born kits (+1.3 kits) than those from the crossing of previous generations, and the response was higher than would have been expected if the heterosis of the crossings had not been modified. This may be due to the zero endogamy of the crossed does and/or the possible complementariness of the responses to selection of the lines used (line A in embryonic survival and V by ovulation rate). These results agree with those reported by Costa *et al.* (2004) when comparing does crossed with selection differences of 13 generations in one of the maternal lines.

As for the effect that selection may have had on the does, when the does are subjected to the same productive pressure (standardised litter), differences in feed ingestion and milk production are observed at the onset of lactation, depending on the selection by litter size. These results could explain a possible change in the use of available resources by the animal as a result of the selection. In this way, when selecting the animals by litter size at weaning, we would select both criteria of prolificacy (and in fact more kits are born) and maternal aptitude criteria

(survival of the kits). Survival in lactation is mainly determined by what happens in the first days after parturition, and is clearly related to the ingestion of energy by the kits in that period. Therefore, the increase in milk production as a result of the greater ingestion of the does would be favourable.

Finally, another point to consider is the possible effect of selection on the productivity and life expectancy of the does in the farm, although there are practically no works in this respect. Theilgaard *et al.* (2005b) recently analysed the risk of elimination, throughout 6 reproductive cycles, of 166 females crossed with different degrees of selection by litter size, using a Cox proportional risk model and the Survival Kit program (Ducrocq and Sölkner, 1998). Although the differences in risk between the two genetic types were not significant (P=0.12), they were considered relevant. The elimination of a doe from the control group was 1.56 times more likely than that of one from the selected group. This result goes against the resources allocation theory that postulates that the more energy used for reproduction the fewer resources will be available for other functions, such as survival (Reznick *et al.*, 2002).

However, it is possible that the selection of the animals on reproductive criteria has produced a response correlated to t he capacity of the animals to obtain resources (van Noordwijk and de Jong, 1986; Reznick *et al.*, 2002). In fact, Quevedo *et al.* (2005) indicated a possible increase in the efficacy of use of energy feed for foetus production as a consequence of selection by reproduction criteria (0.29 and 0.33 for old and current does, respectively). Indeed, the product of gestation was clearly higher in the selected does; although they did not show greater energy ingestion, or a greater mobilisation of reserves.

Conclusions

Throughout this chapter, the latest advances in nutritional techniques and strategies for the handling of reproducing rabbit does have been reviewed, considering productive criteria in the short term (litter size, milk production...), but especially long term criteria such as body condition, life expectancy, etc. The development of techniques monitoring the body condition of reproductive rabbit does has allowed a better knowledge of their evolution throughout the reproductive cycle and the doe effective life, providing knowledge of the most critical moments, and the possible effect of the body condition on reproductive efficacy or longevity. In future, this information will allow the development of global nutritional strategies for reproductive rabbit doe. Some results are already available from feeding programmes in rearing, the use of energy feeds or the adjustment of the nutrition to genetic improvement. Nevertheless, a greater effort must be made in the study of global nutritional strategies taking into account the productivity of the reproductive doe in the long term and the possible effect on farm health.

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4.6. Feed additives to reduce the use of antibiotics

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Introduction

In the rabbit meat production as well as in other animal species, antibiotics have been used for therapy, disease prevention, and production enhancement. The use of antibiotics in animal production, however, has been viewed critically because of their impact on the development of resistant bacteria that compromise human health. Approvals for their routine application are therefore disappearing worldwide and from January 2006 onward, antibiotics for use as growth promoters are forbidden in the EC.

In poultry and pig industry, increasing standards of animal hygiene are applied to enable production models with a low antibiotic use. Although also efforts are done in rabbit meat production, due to e.g. the smaller production units, prevention programs are more difficult in rabbits. Furthermore, the etiology of enteropathies, which are responsible for a significant mortality and morbidity of rabbits after weaning, is often unclear because causes are frequently multiple and some pathogenic agents have not yet been identified (Licois, 2004). The rabbit industry has to face this situation. Besides improved management techniques alternatives to antibiotics are increasingly searched with a view to disease control. Among the candidate replacements for antibiotics are probiotics, prebiotics, organic acids, plant extracts, enzymes and immune modulators.

1. Probiotics

Although microorganisms containing foods (e.g., fermented milk) have been used for millenia, the idea of probiotic is usually credited to Elia Metchnikoff, working at the turn of last century (Gibson, 1999), and the creation of the word generally, although probably wrongly, attributed to Parker (Hamilton-Miller *et al.*, 2003). Probiotic was a term first applied in human nutrition, with the meaning of a microorganism in a food. The concept was later extended to animal nutrition, where it is now considered a particular microbial type of feed additive. Definitions of probiotic have varied somewhat, but it is generally accepted that a probiotic is a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.

Probiotics are used as a possible alternative to antibiotics because they intend to improve (i) the performances and (ii) the health of farm animals. An overview of the results with different probiotics in rabbit production is summarized in Table 1.

A lack of consistency is evident in the performance results, whether we look at average daily gains or feed conversion ratio as well health responses. Inconsistent results have also been obtained with probiotics in other animal species (Doyle, 2001; Simon *et al.*, 2003), a fact which is not surprising given the complexity of the gut ecosystem, where the probiotic is expected to have its effects. The responses to probiotics probably depend on the nature of the probiotic, [the strain, the dose and means of administration] the individual animal, and the environmental conditions.

The mode of action of probiotics remains largely unexplained. Various hypotheses have been proposed, with various possible actions, of variable intensities, at the same time (Bomba *et al.*, 2002). Some of them may have to do with the microbes that

Table 1 The relative improvement in rabbit zootechnical performances with probiotic addition (% difference with the control group)

Probiotic	Rabbits	Average daily gain	Feed conversion	Mortality[2]	References
Lacto-Sacc yeast culture	Growing Diet: 23.1% ADF	NS[1]	NS	No effect	Luick *et al.*, 1992
Lacto-Sacc yeast culture	Growing Diet: 9.9% ADF	NS	NS	No effect	Luick *et al.*, 1992
Lacto-Sacc[3]	Growing, commercial rabbitry	NS	NS	NS	Gippert *et al.*, 1992
Lacto-Sacc	Growing, Exper. rabbitry	NS	No effect	No effect	Gippert *et al.*, 1992
Lacto –Sacc	Growing Rabbit farm	+ 12.5	NS	-	Yamani *et al.*, 1992
Paciflor Bacillus CIP 5832	Growing	+ 6.1	NS	NS	De Blas *et al.*, 1991
Paciflor *Bacillus* CIP 5832	Growing Optimal housing conditions	NS	- 2.4	No effect	Maertens *et al.*, 1994
Paciflor *Bacillus* CIP 5832	Growing Less favourable housing conditions	NS	-	NS	Maertens *et al.*, 1994
Biosaf 0.15% *Saccharomyces cerevisae*	Growing Optimal housing conditions	NS	NS	No effect	Maertens and De Groote, 1992
Biosaf 0.15% *Saccharomyces cerevisae*	Growing Less favourable housing condition	+ 4.0	-	-10.5	Maertens and De Groote, 1992
BioPlus 2B *Bacillus licheniformis Bacillus subtilis*	Growing Summer condition (18 – 26ºC)	No effect	NS	-16.6	Kustos *et al.*, 2004
Lact-A-Bac *Lactobacillus acidophilus*	Growing, Rabbitry farm of University	+9.6	-6.5	No effect	Amber *et al.*, 2004
Bacillus cereus var.toyoi	Growing, Commercial farm	+4.2	- 3.7	NS	Trocino *et al.*, 2005
		Parturition interval %	Litter weight at weaning %	Litter size at weaning %	
Bacillus cereus var.toyoi	Doe rabbit	- 10.2	NS	NS	Nicodemus *et al.*, 2004
Biosaf 0,15% *Saccharomyces cerevisae*	Doe rabbit	-	NS	NS	Maertens and De Groote, 1992
Paciflor *Bacillus* CIP 5832	Doe rabbit	-	+6.4	NS	Maertens *et al.*, 1994

[1] NS: differences from control are not significant (P>0.05) ; [2] % mortality control group - % mortality experimental group ; [3] Micrencapsulated bacteria (*streptococcus faecium* and *lactobacillus acidophilus*) enzymes (protease, amylase and cellulase) and yeast culture (strain 1026)

inhabit the gut of the animal, while some others may; be related to the animal's immune system [an interaction between these two broad classes of effects cannot be ruled out]. According to Simon *et al.* (2003), the possible microbial effects of a probiotic include an increase in the number of beneficial species, a competition with noxious species for epithelial receptors and/or nutrients, the passive absorption of these species, the production of antibiotic-type substances (e.g. bioactive peptides), the production of acids, leading to a reduction in pH, and a reduction in the bacterial deconjugation of bile acids.

Reviews of probiotics in human nutrition stress their effects on the immune system (Oowehand *et al.*, 1999; Rolfe, 2000). Such effects have been mainly studied either *in vitro*, or with the model rodents usually used in the human area (Perdigon *et al.*, 1995; Perdigon *et al.*, 1999). Though outnumbered, some studies of the subject have nevertheless been carried out with farm animals. Recently, Dalloul *et al.* (2003) showed an effect of a probiotic on the intestinal immune system of broiler chickens. In their study, intestinal intraepithelial lymphocyte numbers were increased, and the birds got higher resistance to coccidia (Eimeria acervulina, specifically). In another study with meat-type chickens, Koenen *et al.* (2004), using a *Lactobacillus*-based probiotic, got positive humoral and cellular immune responses. Comparable observations were obtained with pigs. However, Lactobacilli, which are the most common probiotics, are not regular inhabitants of the rabbit digestive tract and poorly adhere to epithelial cells. So their usefulness in rabbits is doubtful (Yu and Tsen, 1993).

2. Prebiotics

Prebiotic oligosaccharides are defined as non-digestible food ingredients that stimulate selectively the growth and (or) activity of potentially health-enhancing intestinal bacteria (Flickinger *et al.*, 2003). They are not digested hydrolytically in the upper intestinal tract of monogastric animals and are thus available for fermentation by the hindgut flora (Fishbein *et al.*, 1988).

2.1. Mannanoligosaccharides

Mannanoligosaccharides (MOS) have shown to have potential to prevent pathogens from colonizing the alimentary tract. The mode of action of MOS is based on competitive exclusion. Exclusion of enteric pathogens may be effected through the use of simple sugars such as mannose, which have been shown to inhibit adherence of pathogenic bacteria in the gastrointestinal tract by blocking bacterial lectin-epithelial receptor interaction. Unfortunately, inclusion of mannose in animal diet would be too expensive. Mannose-containing carbohydrates, however, are present in yeast cell wall and MOS from yeast are available on the market. Several studies showed that MOS are an alternative to antibiotics in poultry (Samarasinghe *et al.*, 2003; Hooge *et al.*, 2003; Sims *et al.*, 2004). MOS improved performance of broiler chickens (Hooge *et al.*, 2003) and turkeys (Sims *et al.*, 2004) equivalent to bacitracin. MOS supplied at a rather high dose of 2.5% to diets for chickens affected the birds' intestinal microflora by increasing *Bifidobacterium* sp. and *Lactobacillus* sp., while decreasing the *Enterobacteriaceae* groups (Fernandez *et al.*, 2002). Mannans derived from the cell wall of *Saccharomyces cerevisiae* increased weight gain, improved feed efficiency and beneficially modulated immune function in the weanling pig (Davis *et al.*, 2004). In rabbits, Pinheiro *et al.* (2004) studied effect of MOS on ileal morphometry and caecal fermentation. MOS were added at 0, 1, 1.5 and 2 g/kg. Histological examination of ileal samples showed significantly longer villi and higher total volatile fatty acid concentration in the caecum of rabbits fed MOS. Furthermore, caecal pH was lower and caecal butyric acid concentration higher in rabbits fed MOS. Fonseca *et al.* (2004) compared effects of dietary MOS and oxytetracyclin on performance of growing rabbits. MOS and oxytetracyclin were added at 2 g/kg and 0.2 g/kg to the grower and finisher diet, respectively. Effect of both additives on overall weight gain was similar. Mortality was significant lower in rabbits fed the diet with MOS than in those fed the diet supplemented with oxytetracyclin (6.3 vs 11.9%, respectively). Contrary to this, Scapinello *et al.* (2001) found no significant effect of MOS addition on rabbit performance. The number of rabbits per group, however, was small (10 per a treatment) in their experiment.

2.2. Other oligosaccharides

Other oligosaccharide-based prebiotics, such as the fructo-oligosaccharides (FOS), gluco-oligosaccharides (GOS) and inulin have also been tested in rabbits. Again, results were inconsistent, whether one looks at performances or caecal fermentation pattern. Lebas (1996) got a FCR response (with FOS), without a change in ADG while Aguilar *et al.* (1996) found the opposite. Luick *et al.* (1992) and Mourão *et al.* (2004) could not get any significant effect of dietary FOS addition. Morisse *et al.* (1992) obtained with a dietary addition of FOS a beneficial effect on the rabbit's resistance against an *E. Coli* challenge. Performances of fatteners on GOS supplemented diets were not significantly different (Gidenne 1995: Peeters *et al.*, 1992). However, Gidenne (1995) observed a significant increase in morbidity and mortality.

Dietary additions of GOS or FOS affected the caecal fermentation pattern in some studies (Morisse

et al., 1990, 1993; Peeters *et al.*, 1992), whereas no effect was found in other experiments (Lebas, 1993; Gidenne, 1995). In inulin-fed rabbits a reduced caecal pH and increased volatile fatty acids concentrations (Volek *et al.*, 2005) or butyrate proportions were found (Maertens *et al.*, 2004). Moreover structural changes in the gut architecture were observed (Alves *et al.*, 2003).

3. Organic acids

Organic acids (acetic, lactic, benzoic, sorbic) have a long history of use in the food industry as food preservatives. The mode of action of organic acids against micro-organisms is not fully understood. It has been assumed that undissociated forms of organic acids penetrate the lipid membrane of the bacterial cell and dissociate into anions and protons inside. As bacteria maintain a neutral pH of the cytoplasm, the export of excess protons consumes cellular ATP and results in depletion of energy (Ricke, 2003). Unlike antibiotics, the antimicrobial activity of organic acids is thus pH dependent. In cultures of *Escherichia coli* treated with caprylic acid at pH 5.2 the number of viable cells decreased to ca 102/ml. A reduction of a mere 0.94 - 1.96 log10 cuff was observed at pH 6.5 - 6.6 (Marounek *et al.*, 2003). The lowering of dietary pH alone, however, failed to show any nutritive efficacy (Roth and Kirchgessner, 1998). Supplementation of diets of growing rabbits with commercial acidifiers did not affect any performance characteristic (Scapinello *et al.*, 2001).

Formic, fumaric and citric acid have a beneficial effect on growth and feed-to-gain ratio in weaned piglets and fattening pigs (Partanen and Mroz, 1999). There are also studies, though less numerous, testing performance effects of feeding propionic, malic, sorbic, tartaric, lactic and formic acid in poultry (reviewed by Dibner and Buttin, 2002). Reports on the use of organic acids in the nutrition of rabbits are scarce. Fumaric acid had no effect on weight gain, but improved the feed/gain ratio when added to the diet of rabbits at 2.0% (Scapinello *et al.*, 2001). Caprylic acid at 5 g/kg feed had no effect on the rate of growth, but decreased mortality in the post-weaning period (Skřivanová and Marounek, 2002). Rabbit gastric and pancreatic lipase activities are high enough to release caprylic and capric acid from their ester bonds. Thus, triacylglycerols of both medium-chain fatty acids represent a similar feed additive with antimicrobial activity (Skřivanová and Marounek, 2006).

4. Plant extracts

Plant extracts and various products containing essential oils are among the alternatives of antibiotics that are already used in practice. Oregano, savoy and thyme contain compounds with broad antibacterial activity such as thymol, carvacrol, p-cymene and γ-terpinene (Nevas *et al.*, 2004). In chickens, a commercial preparation of essential oils reduced the concentration of *Clostridium perfringens* in the intestine (Losa and Köhler, 2001), but failed to improve growth performance (Lee *et al.*, 2003). In a rabbit experiment, mint extract surpassed anise extract with regard to the effect on performance of rabbits reared in hot climate conditions (Rashwan *et al.*, 1996). Botsoglou *et al.* (2004) showed that oregano essential oil exerted no growth-promoting effect on rabbits, but significantly improved oxidative stability of rabbit meat.

5. Enzymes

Viscous polysaccharides are thought to drag digesta to the distal part of the intestines, providing the substrate for microbial proliferation. It has been shown in weaned piglets that increasing the viscosity of the intestinal contents stimulate growth of enterotoxigenic strains of *Escherichia coli* (McDonald *et al.*, 2001). Thus, polysaccharidases (xylanase, pectinase, β-glucanase) can influence proliferation of enteropathogenic bacteria in the intestine by reducing the digesta viscosity, making nutrients available for absorption before reaching fermentative parts of the digestive tract. Similarly in chickens a significant predisposing factor to outbreak of clostridial infection is the level of crude protein in the diet (Drew *et al.*, 2004). Presumably, at high level of dietary protein more protein might reach the caeca and increase the number of clostridia. Indeed, supplementation of a diet of weaned rabbits with protease and xylanase decreased mortality of rabbits without an effect on performance traits (García *et al.*, 2004). Enzyme preparation containing amylase activity reduced ileal starch concentration (Gutiérrez *et al.*, 2002) and mortality when the incidence of intestinal disorders was of relevance (Cachaldora *et al.*, 2004).

6. Concluding remarks

Contrary to pigs and poultry, studies dealing with alternatives to antibiotics are quite limited in rabbits. Apparently, several alternatives to antibiotics exist, and at present only limited information about e.g. their efficiency, conditions of use and mode of action is available in scientific literature. Probably, many studies remain unpublished because of confidentiality, either because of favourable (protection for use with license …) or unfavourable results. Oligosaccharides have been the most intensively studied in rabbits. Plant extracts are complex mixtures of compounds with antimicrobial and/or other physiological activity. It can not be excluded that some of them negatively influence the intermediary metabolism of

the animal. A thorough examination of plant extracts *in vitro* and in vivo is necessary before their use on a large scale. In addition, it should be kept in mind that the increase of resistance of microbes to above mentioned antimicrobials is unavoidable after their long-term application.

The number of mechanisms of defence of microorganisms against antimicrobial compounds is limited (Hogan and Kolter, 2002). The long-lasting use of non-antibiotic antimicrobials on a large scale may cause the spread of resistant microorganisms in the environment, with consequences similar to those that led to the antibiotic ban. Exogenous polysaccharidases can influence microbial growth in the intestine in spite of the absence of antimicrobial activity. Enzyme supplements might be effective in young animals with their less developed digestive system.

Finally, further research is necessary to study the combined use of pro- and prebiotics. Prebiotics can probably stimulate the colonisation of probiotics. Even a combination of pre- and probiotics with organic acids could have potential to increase gut health.

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Chapter 5

RABBIT MEAT QUALITY AND SAFETY

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Rabbits are sold as a whole carcass, retail cuts, and processed meat products ready to cook, thus many attributes of both carcass and meat quality have to be considered. The most frequently measured traits of body composition according to the norms of the World Rabbit Science Association (Blasco and Ouhayoun, 1996), are dressing out percentage, proportions of fore, intermediate and hind parts in the chilled carcass, carcass fatness estimated by perirenal or dissectible fat weights relative to carcass weight, and muscle to bone ratio assessed in the hind leg as the best predictor of the meat:bone ratio in the carcass. In addition, leg bone resistance may be considered as another quality factor for both live animals and sold carcasses.

Meat quality is a complex concept, that is usually assessed by a mixture of chemically and histologically determined muscle traits, instrumentally measured raw or cooked meat characteristics, and scores attributed by a trained taste panel. It classically comprises water-holding capacity, color assessed on fresh-cut muscle surface, cooking loss, and pH measured on the day after slaughter as a value that predicts technological and eating quality of the meat. According to recent data, sensory properties are also main criteria influencing consumer's choice (Dalle-Zotte, 2002), especially regarding tenderness and flavor. Both instrumentally-measured texture and sensory evaluation are used to evaluate rabbit meat tenderness. In addition, content and composition of intramuscular fat have been considered as important factors for eating quality, although correlations between fat content in the rabbit meat and cooking losses or sensory characteristics remain somewhat controversial (Gondret *et al.*, 1998; Hernandez *et al.*, 1998; 2000). Finally, the number, size, and type frequency of the muscle fibers at slaughter could also prevail in meat sensory quality.

Traceability is now emerging as a "catch-word" for consumer and regulatory confidence with respect to food quality, food safety and the infrastructure for producing, processing food product from the point of origin to point of sale. The need for traceability has arisen primarily from consumer and government concerns over food safety, hygiene and authenticity. However the driving force behind the introduction of traceability in the food sector has been a combination of recent food scandals and the wish of authorities to protect the health of the consumer by reducing the risk from the food that they eat.

Numerous crises including Bovine Spongiform Encephalitis (BSE) in bovine, high dioxin levels in chicken coming from illegal use of transformer oil in swine, the danger of increased spread of other infectious disease (e.g. Foot and Mouth disease, Avian influenza, etc.), as well as pathogens such as salmonella, listeria, clostridium and E. coli 0157 have recently hit the European livestock and meat chains. Another perceived risk is associated to the consumption of meat deriving from animals that were genetically modified or were fed a diet containing genetically modified organism (GMOs)

(McGrann and Wiseman, 2001; Morrison, 2003; Miraglia *et al.*, 2004; Lupien, 2005).

These concerns attracted massive media attention and led to a decline in consumer confidence and subsequent financial losses for the meat industry. Since the emergence of these crises, consumers are demanding traceability at retail and catering levels for all animal products. This demand extends to the farm of origin and includes the feedstuffs, feed ingredients, and the chemicals administered to the animals or to which the animals are exposed. Recent research has shown that consumers have considerable difficulties in forming meat quality expectations (Grunert *et al.*, 2004).

The need for traceability has also arisen for consumer concerns over food authenticity. It is well-known that organic foods, protected designation of origin (PDO) and protected geographical indication (PGI) products and other labels are steadily gaining in popularity. A category of consumers is looking for traditional, regional, handmade products which are supposed to be safer because they are more natural. If one wants to keep consumers' confidence in such labels, the early detection of any kind of fraud must be made possible.

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5.1. Rabbit meat quality

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1. Nutritional quality of rabbit meat

The nutritive value of meat has an increasing importance among the factors determining meat quality and consumer acceptability. Indeed, meat is a major source of proteins and essential amino-acids. However, meat is also a major source of saturated fatty acids, for which a high consumption may be related to chronic non-deficiency diseases, e.g., obesity, type2 diabetes and cardiovascular diseases. Recently, the nutritional value of rabbit meat has been reviewed for several authors (Combes, 2004; Dalle Zotte, 2004; Combes and Dalle Zotte, 2005), showing that rabbit meat has a high nutritional value compared with other meats.

1.1. Chemical composition of rabbit meat

The main components of meat, excluding water, are proteins and lipids. Furthermore, meat is also an important source of highly available micronutrients, such as vitamins and minerals. Although nutrient losses could be produced during cooking, the change in nutritional value of rabbit meat remains small (Dal Bosco *et al.* 2001). Raw rabbit meat is characterised by its lower energetic value (on average 618 kJ/100 g fresh meat) compared with red meats, such as beef and lamb (Dalle Zotte, 2004).

The information available about chemical composition of rabbit meat is extremely variable, especially regarding fat content, depending on the part of the carcass studied (Pla *et al.*, 2004) and also on the different productive factors (Dalle Zotte, 2002). Generally, there is an increase in protein and fat contents and a decrease in water content with increasing age (Gondret *et al.*, 1998a, b; Hernández *et al.*, 2004) and weight (Szendrö *et al.*, 1996) of the animals. Chemical composition of rabbit meat can be also affected by the genetic line (Pla *et al.*, 1998, Hernández *et al.*, 1998), but it is scarcely influenced by gender (Pla *et al.*, 1996, Gondret 1998). Finally, feeding factors have a strong influence on the chemical composition of rabbit meat, in particular on its lipid composition (Dalle Zotte, 2002).

Table 1 shows the chemical composition of rabbit meat from different carcass portions and from the whole reference carcass. The lowest value for protein content corresponds to the thoracic cage (18.7 g/100 g edible meat), while the highest protein content corresponds to the meat of loin and hind leg (22.1 and 21.2 g/100 g edible meat, respectively). Rabbit meat contains high levels of essential amino-acids (Table 2), constituting proteins with a high digestibility value (Bodwell and Anderson, 1986).The fat content varies widely depending of the carcass portion considered (Table 1). Dalle Zotte (2004) gives values of rabbit fat content from 0.6 to 14.4 % with an average value of 6.8 %. The loin was the leanest part of the carcass (1.2 g of lipids/100 g edible meat). The hind leg has a moderate amount of fat (3 g/100 g edible meat). In contrast, the fattest part of the carcass is thoracic cage (12.8 g/100 g edible meat), because includes the neck with high intramuscular fat depots. Besides, intramuscular lipid content varies widely with the muscle site and muscle type considered (Alasnier *et al.*, 1996)

Finally, rabbit meat is particularly tender as a consequence of its lower content of elastine (Ouhayoun and Lebas, 1987) and the high solubility of its collagen comparing with meat from other species (Combes *et al.*, 2003).

1.2. Fatty acid composition and cholesterol

Meat fat contains several types of lipids, including triglycerides as the main components, phospholipids and cholesterol. Contrary to triglycerides which represent the main variable components in lipids as discussed above, the concentration of phospholipids is relatively constant

Table 1. Chemical composition of rabbit meat depending of carcass portions. [a]

Trait	Nutrient	M	SD	Range	VC x 100
Fore legs	Crude protein	20.2	0.72	19.0-22.0	3.57
	Crude fat	7.43	2.76	3.12-13.8	37.2
	Moisture	71.2	3.23	63.9-76.9	4.53
Thoracic cage	Crude protein	18.7	1.19	16.0-20.8	6.37
	Crude fat	12.8	4.97	4.90-22.3	38.8
	Moisture	66.9	4.88	57.0-76.1	7.30
Muscles LD	Crude protein	22.1	0.59	20.8-23.0	2.67
	Crude fat	1.20	0.36	0.62-1.94	30.0
	Moisture	75.6	0.89	73.8-77.9	1.18
Abdominal walls	Crude protein	20.9	0.75	19.5-22.6	3.59
	Crude fat	7.56	3.84	2.21-19.7	5.08
	Moisture	70.1	4.38	57.2-77.0	6.24
Spine	Crude protein	20.7	0.69	19.0-22.2	3.34
	Crude fat	7.93	4.33	2.02-23.2	54.6
	Moisture	70.0	4.68	53.6-77.0	6.68
Hind leg	Crude protein	21.2	0.49	20.4-22.5	2.31
	Crude fat	3.03	1.01	1.32-6.10	33.3
	Moisture	74.7	1.28	71.9-77.0	1.71
Reference carcass	Crude protein	20.8	0.51	19.7-21.9	2.45
	Crude fat	7.09	2.82	2.01-13.3	39.8
	Mistureo	71.2	3.06	64.4-76.8	4.30

LD = *longissimus dorsi*; M = mean (g / 100 g edible meat); SD = standard deviation;
VC = variation coefficient [a]Adapted from Pla *et al.* (2004)

in skeletal muscle, with amounts between 0.5 and 1g/100g of muscle, depending of the metabolic muscle type (Alasnier *et al.*, 1996). Rabbit meat fat comprises mostly saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs), with percentages around, 36.9% and 34.6% of total fatty acids in the hind leg, respectively. Monounsaturated fatty acids (MUFAs) are less represented (around 28.5%, Table 3).

The most ubiquitous fatty acids are oleic (C18:1), palmitic (C16:0), and linoleic (C18:2) acids, showing percentages higher than 20% of total fatty acids. Altogether, rabbit meat has a high ratio of PUFA to SAT fatty acids (0.75 and 0.85 for the loin and the meat of hid leg, respectively; Alasnier *et al.*, 1996; Ramírez *et al.*, 2005). Among the PUFAs, linoleic (C18:2) and linolenic (C18:3) are essential fatty acids, because of animal organisms are unable to synthesize them. Linoleic acid is the precursor of ω6 family of PUFA, while linolenic acid serves the same function for the ω3 family, especially for eicosapentaenoic EPA and docosahexaenoic DHA fatty acids. A minimum intake of combined EPA and DHA of 500 mg/day is recommended for human cardiovascular health (ISSFAL, 2004). The amount of linoleic fatty acid is around ten times greater in rabbit meat than in beef and lamb and around double than the quantity reported for pork meat (Enser *et al.*, 1996). The amount of linolenic acid is also remarkably abundant in rabbit meat (3%) in comparison with those reported in other meats (1.37 in lamb, 0.70 in beef and 0.95 in pork; Enser *et al.*, 1996). Although rabbit meat has a very low amount of EPA and DHA (Ramírez *et al.*, 2005), Dal Bosco *et al.* (2004) have confirmed the ability of rabbit tissues to synthesise these fatty acids from the dietary linolenic precursor. Altogether, the ratio of ω6 to ω3 fatty acids reaches 7 (Dal Bosco *et al.*

Table 2. Aminoacid composition of rabbit meat. [a]

Aminoacids	Average (mg/100 g of edible meat)
Lysine	1.85
Methionine-Cystine	1.10
Histidine	0.53
Threonine	1.16
Valine	0.99
Isoleucine	0.99
Leucine	1.81
Arginine	1.23
Tyrosine	0.73
Phenylalanine	1.03
Tryptophan	0.21

[a] Adapted from Dalle Zotte (2004)

Table 3. Least square means and standard errors of fatty acids content in rabbit leg meat (mg/100g of meat).[a]

Fatty acids	Mean
C10:0 (Capric)	3.19 ± 1.01
C12:0 (Lauric)	6.27 ± 0.68
C14:0 (myristic)	67.1 ± 2.82
C16:0 (palmitic)	712 ± 24.6
C16:1 *cis* ω*7* (palmitoleic)	78.0 ± 5.16
C16:1 *cis* ω*9*	9.36 ± 0.36
C17:0 (Margaric)	16.9 ± 0.63
C17:1 (Heptadecenoic)	6.74 ± 0.58
C18:0 (stearic)	185 ± 5.88
C18:1 ω*9* (oleic)	635 ± 24.3
C18:1 ω*7* (vaccenic)	34.9 ± 1.32
C18:2 ω*6* (linoleic)	777 ± 33.2
C18:3 ω*3* (α-linolenic)	81.2 ± 4.81
C20:1 (icosaenoic)	9.96 ± 0.73
C20:2 ω*6* (eicosadienoic)	12.8 ± 0.58
C20:3 ω*6* (eicosatrienoic)	6.68 ± 0.54
C20:4 ω*6* (arachidonic)	45.4 ± 1.24

[a] Adapted from Ramírez *et al.* (2005)

Table 4. Vitamin composition of rabbit meat. [a]

Vitamins	Average
A (Retinol) μg	Trace
E (Tocopherol) mg	0.186
B1 (Thiamine) mg	0.082
B2 (Riboflavin) mg	0.125
B3 (Niacine) mg	9.6
B5 (Pantothenic acid) mg	0.6
B6 (Pyridoxine) mg	0.34
B8 (Biotine) μg	0.7
B9 (Folic acid) μg	5
B12 (Cobalamin) μg	6.85

Amounts per 100 g of fresh meat [a] Adapted from Combes (2004)

2004) or 11 (Ramírez *et al.*, 2005) for the loin and the meat of hind leg, respectively. Therefore, decreasing the ω6: ω3 ratio up to 5 is an interesting goal to improve the nutritional value of rabbit meat for human benefits (ISSFAL, 2004). In this sense, it is possible to increase the ω3 PUFA level and to decrease the ω6: ω3 ratio trough diet, as we will discus later on.

In addition to fatty acids, cholesterol is a nutritionally important component of meats. The amount of cholesterol of rabbit meat is around 59 mg/100 g of muscle (Combes, 2004), and some muscles as *Longissimus dorsi* and *Psoas major* have even lower values (45 and 50 mg/100g of muscle, respectively, Alasnier *et al.*, 1996). These values are lower than those presented in meat from other species (61 mg in pork, 70 mg in beef, 81 mg in chicken, Dalle Zotte, 2004).

1.3. Minerals and vitamins

In contrast to many plants, meats contain no substances that could interfere with the digestion and absorption of specific minerals (Bodwell and Anderson 1986). The mineral fraction of rabbit meat is characterised by low contents in sodium (49 and 37 mg/100g for hind leg and loin, respectively) and iron (1.3 and 1.1 mg/100g for hind leg and loin, respectively), while the phosphorus level is high (230 and 222 mg/100g for hind leg and loin, respectively; Combes, 2004). Although meat represents the main dietary source of highly available iron, it is important to know the respective amounts of heme and non-heme iron. Indeed, the latter is less readily absorbed than the former iron form. Furthermore, cooking processes can transform heme into non-heme iron, and then change total iron availability. Heme iron represents about 56% of total iron in rabbit raw meat (Lombardi-Boccia *et al.*

2002), and cooking causes a 7% decrease in the heme iron content (Lombardi-Boccia *et al.* 2002). Lombardi-Boccia *et al.* (2005) have also analysed the content of copper and zinc of different types of meats. These authors found that rabbit meat has the lowest zinc concentration (0.55 mg/100g) among all the samples analysed, and that copper concentration is quite similar to the other species analysed (0.03 mg/100g). Skřivanová *et al.* (2002) found copper concentrations of 0.048 and 0.035 mg/100 g in the hindleg and loin of rabbit, respectively. In general, rabbit meat showed a high retention of those minerals after cooking (Lombardi-Boccia *et al.* 2005). Selenium levels in animals depend on their diet, in rabbit meat the values of selenium concentration offered in literature vary between 9 µg/100g (Díaz-Alarcon *et al.*, 1996) and 22 µg/100g (Wiesner *et al.*, 1978).

Table 4 shows the vitamin composition of rabbit meat (Dalle Zotte, 2004). Meat is an important source of B vitamins. Consumption of 100g of rabbit meat contributes to 8% of daily B2 vitamin, 12 % of B5 vitamin, 21% of B6 vitamin, 77% of B3 vitamin requirements, and provides a fulfilment of daily B12 vitamin requirement (Combes 2004). Contrary to what is observed for mineral elements, heat treatments alter B vitamins content. B1 vitamin reaches undetectable level in rabbit cooked meat, whereas complete or high retention for B2 and B3 vitamins, respectively, is seen after cooking (Lombardi-Boccia *et al.* 2005). In addition, rabbit meat as other meats, contains only trace amounts of A vitamin. Nevertheless, it should be noticed that a high amount of this vitamin can be found in rabbit edible liver (Ismail *et al.*, 1992). Extra supplementation of E vitamin in the diet (200 mg/kg) to improve the oxidative stability of the meat, led to an increase of almost 50% of E vitamin in rabbit meat (Castellini *et al.*, 2000). Besides, E vitamin content is not affected by cooking treatment (Dal Bosco *et al.*, 2001).

1.4. Conclusion

In general, the information about mineral and vitamin content in rabbit meat remains scarce. The inclusion of rabbit meat in the human diet may however improve human health, since it is a lean meat rich in proteins of high biological values, with highly unsaturated lipids, low cholesterol content, and noticeable quantities of linolenic fatty acid (C18:3 ω3). Besides, it displays a low content of sodium and a high content of phosphorus, and may be a good source of B vitamins.

2. Effect of genetic selection on rabbit meat quality

Meat rabbit selection programs are usually based on a three-way cross. Two breeds are selected for litter size to produce the crossbred dams, and a large size terminal sire line is used in terminal cross. For the last 15 years, selection of buck lines for growth rate has been largely successful (Larzul and Gondret, 2005 for a review). Whatever the genetic selection aimed at improving ADG or live weight at a fixed age, similar responses to selection have been obtained on these two criteria. Altogether, post-weaning ADG might be improved in the range of 0.45 to 1 g/d per generation of selection, depending of the experiments (Rochambeau *et al.*, 1989; Piles and Blasco, 2003; Larzul *et al.*, 2005). Even when ADG was included in a combined selection index with reproductive traits, improvement in growth rate has been clearly shown (Moura *et al.*, 2001; Garreau and Rochambeau, 2003). Increasing post-weaning ADG is also an indirect form of improving feed efficiency, because of the negative genetic correlation between these two criteria (Moura *et al.*, 1997; Piles *et al.*, 2004). However, selection for an improved growth rate may have adverse effects on quality traits. The aim of this subchapter is to present the last experiments about genetics of carcass and meat traits in rabbits.

2.1. Genetic variation on carcass and meat quality

It is well-known that selection for ADG led to increased body weight along the growth curve, including mature size (Blasco *et al.*, 2003). Body weight at a fixed age was moderately heritable in rabbits, since heritability values (h^2) fell within the range of 0.12 to 0.67 (Camacho and Baselga, 1990; Ferraz *et al.*, 1991; Estany *et al.*, 1992; Rochambeau *et al.*, 1994; Lukefahr *et al.*, 1996; Gomez *et al.*, 1998; Anous, 1999; Farghali and El Mahdy, 1999; Garreau *et al.*, 2000; Moura *et al.*, 2001; Piles and Blasco, 2003; Larzul *et al.*, 2003, 2005; Larzul and Rochambeau, 2005). Similarly, h^2 for ADG ranged of 0.17 to 0.29 (Rochambeau *et al.*, 1989; Lukefahr *et al.*, 1996; Piles and Blasco, 2003; Piles *et al.*, 2004; Larzul *et al.*, 2005). In addition, body weight at slaughter age and ADG were generally strongly associated, with a genetic correlation coefficient comprised between 0.75 to 0.98 (Szendrö *et al.*, 1988; Lukefahr *et al.*, 1996; Garreau *et al.*, 2000; Piles and Blasco, 2003; Larzul *et al.*, 2005).

Table 5 shows the scarce number of published estimates of heritability and genetic parameters of quality traits in the rabbit. Genetic correlation coefficients among body or carcass traits were very variable, and all of them displayed high standard errors due to the small size of samples under evaluations. Moderate to high h^2 have been given for dressing out percentage (Lukefahr *et al.*, 1996; Su *et al.*, 1999, Larzul *et al.*, 2005), with an average value closely similar to that found in the pig (Sellier, 1998). Genetic correlations between body weight at a fixed age and dressing out were small (Lukefahr *et al.*, 1996; Farghaly and El-Mahdy, 1999; Su *et al.*, 1999; Larzul *et al.*, 2005). The h^2 for perirenal fat

Table 5. Average values of heritability (h^2) and genetic correlation (r_g) with mass weight, for carcass and meat quality traits.

Traits	Average h^2	Range of estimates (number of studies)	Averagere	Range of estimates (number of studies)
Body weight at slaughter age	0.27	12-0.67 (17)	-	-
Dressing out	0.34	0.17-0.56 (3)	0.34	0.09-0.56 (3)
Fat percentage	0.64	- (1)	0.24	- (1)
Loin cut yield	0.22	0.19-0.25 (2)	0.52	- (1)
Muscle:bone ratio	0.31	0.23-0.37 (4)	0.78	0.70-0.85 (2)
Colour (surface)				
L*	0.01	(1)	-	-
a*	0.01	(1)	-	-
b*	0.01	(1)	-	-
Ultimate pH	0.16	(1)	0.02	(1)
Maximum shear force	0.57	(1)	0.02	(1)

proportion in the carcass seemed to be relatively high, but this value has been estimated in one experiment only (Larzul *et al.*, 2005). The same authors also reported a positive genetic correlation between weight at a fixed age and the proportion of fat in the carcass. A high correlation coefficient has been found between body weight at a fixed age and muscle-to-bone ratio, falling into the range 0.70-0.85 (Lukefahr *et al.*, 1996; Farghaly and El-Mahdy, 1999). A recent study (Larzul *et al.*, 2005) has also provided genetic estimation for rabbit meat quality traits. Ultimate pH in the loin seemed to have a low heritability, and the authors also reported a null correlation between weight at 63 days of age and pH. Calculated heritabilities for carcass color traits were zero (Larzul *et al.*, 2005), whereas genetic correlation between growth performance and muscle myoglobin content was weakly negative (-0.09 to -0.25; Ouhayoun and Dalle-Zotte, 1993 for a review).

Most values have to be confirmed in a larger set of animals and experiments. For the moment, it is however interesting to note that no evidence for major genes influencing carcass traits and (or) meat quality was provided in the rabbit.

2.2. Phenotypic variations on carcass and meat quality at a fixed age

It is generally considered that most of the phenotypic responses observed in growth-selected animals are achieved as a consequence of changes in mature size (Taylor, 1985). Growth-selected rabbits have approximately the same degree of maturity than unselected animals when slaughtered at the same age (Blasco *et al.*, 2003). Then, numerous investigations for carcass composition and meat traits in response to growth rate have been made at a fixed age, by comparing different breeds (e.g., Skřivanová *et al.*, 1995; Lambertini *et al.*, 1996) or lines selected on different objectives (e.g., growth rate and maternal characters; Deltoro and Lopez, 1986; 1987). Differences observed in these studies were probably due to the genetic origin of the animals rather than to growth performance. Within-breed selection experiments have also been recently performed. They included rabbits selected for a rapid growth compared with a control population, as well as divergent lines selected for body weight at 63-days of age compared with a cryo-preserved group.

Table 6 shows the main results obtained on carcass composition and meat quality traits in within-breed selection experiments. Generally, growth-selected animals and controls displayed a similar dressing out percentage at a same age. Higher, similar, or lower fat percentages have been found in the carcasses of growth-selected animals compared with controls. Depending on the generation of selection, both a higher or similar muscle:bone ratio have been evidenced in growth-selected rabbits compared with controls. In mammals, it was generally assumed that selection for rapid growth and improved muscularity turns metabolism toward an enhanced muscle glycolytic pattern during life, thus leading to a lower ultimate pH in the meat (Ashmore *et al.*, 1972). Regarding within-breed selection experiments, it is first interesting to note that no pH-related abnormalities such as PSE-like syndrome still appeared in growth-selected rabbits. Some experiments even found a higher ultimate pH in the longissimus muscle of

Table 6. Influences of within-breed selection for growth performance on carcass merit and meat quality traits in rabbits at a fixed age.

Trait	Phenotypic evolution	Generation	Type of selection	References
Dressing out	F = S	G3	Divergent selection for fast (F) vs.	Gondret *et al.*, 2002
	F = C > S	G6	slow (S) growth rate, and a control	Larzul *et al.*, 2005
			group (C)	
	F = C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	F = C	G11	control group (C) of a former	Hernández *et al.*, 2004
	F = C	G14	generation	Pascual *et al.*, 2004
Muscle:bone ratio	F = C = S	G6	Divergent selection for fast (F) vs. S growth rate, and a control group (C)	Larzul and Gondret, 2005
	F = C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	F > C	G11	control group (C) of a former	Hernández *et al.*, 2004
	F = C	G14	generation	Pascual *et al.*, 2004
Carcass fatness	F = S	G3	Divergent selection for fast (F) vs. S	Gondret *et al.*, 2002
	F = C > S	G6	growth rate and a control group (C)	Larzul *et al.*, 2005
	F < C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	F < C	G11	control group (C) of a former	Hernández *et al.*, 2004
	F > C	G14	generation	Pascual *et al.*, 2004
WHC[3]	F < C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	F < C	G11	control group (C) of a former	Hernández *et al.*, 2004
	F < C	G14	generation	Ramírez *et al.*, 2004
Muscle CIE L*[1]	F = C = S	G6	Divergent selection for fast (F) vs. S growth rate, and a control group (C)	Larzul *et al.*, 2005
	S = C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	S = C	G11	control group (C) of a previous	Hernández *et al.*, 2004
	S = C	G14	generation	Ramírez *et al.*, 2004
Ultimate pH[1]	F = S	G3	Divergent selection for fast (F) vs. S	Gondret *et al.*, 2002
	F = C = S	G6	growth rate and a control group (C)	Larzul *et al.*, 2005
	F = C	G8	Selection for fast (F) growth rate vs. a	Piles *et al.*, 2000
	F > C	G11	control group (C) of a previous	Hernández *et al.*, 2004
	F = C	G14	generation	Ramírez *et al.*, 2004
Muscular energy metabolism[1]	F = C	G11	Selection for fast (F) growth rate vs. a	Hernández *et al.*, 2004
	F = C	G14	control group (C) of a previous generation	Ramírez *et al.*, 2004
Fiber type frequency	F = S[2]	G3	Divergent selection for fast (F) vs. S	Gondret *et al.*, 2002
	F = C = S[2]	G6	growth rate, and a control group (C)	Larzul *et al.*, 2005

Trait	Phenotypic evolution	Generation	Type of selection	References
	F < C[1]	G14	Selection for fast (F) growth rate vs. a control group (C) of a previous generation	Ramírez *et al.*, 2004
Meat fat content	F = S[2] F = C > S[2] F = C = S[1]	G3 G6	Divergent selection for fast (F) vs. S growth rate, and a control group (C)	Gondret *et al.*, 2002 Larzul *et al.*, 2005
	F < C[3] F < C[3] F > C[3]	G8 G11 G14	Selection for fast (F) growth rate vs. a control group (C) of a previous generation	Piles *et al.*, 2000 Hernández *et al.*, 2004 Ramírez *et al.*, 2005
Maximum shear force	F > S[2] F > S[1]	G3	Divergent selection for fast (F) vs. S growth rate	Gondret *et al.*, 2002
	F = S[2] F < C = S[1]	G6	Divergent selection for fast (F) vs. S growth rate, and a control group (C)	Larzul *et al.*, 2005
	F = C	G14	Selection for fast (F) growth rate vs. a control group (C) of a former generation	Ramírez *et al.*, 2004
Meat sensory tenderness	F = C	G14	Selection for fast (F) growth rate vs. a control group (C) of a former generation	Hernández *et al.*, 2005

[1]Measured in the longissimus muscle (loin) [2]Measured in the semitendinosus muscle (thigh)
[3]Measured in hind leg homogenate

growth-selected animals compared with controls (Hernández *et al.*, 2004), although this increase was not confirmed in the latter generations of selection (Ramírez *et al.*, 2004). The lack of variation in ultimate pH in response to selection was support by the absence of changes in muscle energy equilibrium at the time of slaughter, as assessed as the ratio of glycolytic to oxidative enzyme activities (Table 6). The frequency of the different fiber types in a thigh muscle did not change with selection (Larzul *et al.*, 2005), but a slightly lower percentage of myosin heavy chain (MHC)-I representative of slow-twitch oxidative myofibers was evidenced in the loin meat of growth-selected rabbits (Ramírez *et al.*, 2004).

Meat color is another important parameter that is partly related to ultimate pH. As shown in Table 6, meat lightness of fresh-cut muscle surface remained unchanged between growth-selected rabbits and the control group. On the opposite, the different experiments showed various results concerning a* (redness) and b* (yellowness) values in response to selection (Gondret *et al.*, 2002; Hernández *et al.*, 2004; Ramírez *et al.*, 2004). Water holding capacity of the raw meat was generally poorer in growth-selected rabbits compared with a control group (Table 6). However, this change was not sufficient to lead to any variations in cooking losses (Piles *et al.*, 2000).

In many species, selection for improved growth rate generally favors both the hyperplasia (increase in total number) and hypertrophy (increase in cross-sectional area) of the myofibers (Rehfeldt *et al.*, 2000 for a review). In contrast, there was no variation in total fiber number assessed in the semitendinosus muscle between rabbits showing divergent growth rate (Gondret *et al.*, 2002). This lack of selection-induced difference in total fiber number might be attributed to the moderate genetic progress obtained yet for ADG in rabbit experiments relative to other species (mice and chicks). Conversely, rapid growing rabbits displayed enlarged cross-sectional areas of myofibers, leading to higher muscle weights when compared to slow growing rabbits at a same age (Larzul *et al.*, 2005). However, in this latter experiment, muscles from fast-growing animals and those from controls displayed similar histological characteristics. Both the number and size of myofibers are though to have an influence on meat tenderness. Instrumentally, the loin meat of growth-selected rabbits was found

harder than controls (Ramírez *et al.*, 2004; Gil *et al.* 2006). Nevertheless, trained panel analyses did not detect any differences in meat tenderness between growth-selected rabbits and the control group (Hernández *et al.*, 2005). Besides, selection for growth rate did not affect the activities of proteolytic enzymes and their inhibitors (Gil *et al.*, 2006).

Selection-related changes in muscle fat content generally followed variations reported in carcass dissectible fat deposits (Table 6). Selection for growth rate also seemed to affect the composition of muscle fat at slaughter, with higher proportions of myristic and palmitic acids and lower percentage of linoleic and arachidonic acids in growth-selected animals compared with controls (Ramírez *et al.*, 2005). However, changes of indices related to human health were very small (Ramírez *et al.*, 2005).

No differences have been found between growth selected rabbits and controls for lipolytic enzymes activities (Ariño *et al.*, 2003). Rabbit meat is often negatively perceived by young consumers because of its wild game meat flavor (Dalle-Zotte, 2002), which may partially result of fatty acid composition. It may be considered that selection for growth rate had a negative effect on rabbit meat flavor, with a less pronounced aniseed aromatic note and a higher liver flavor attributed to the meat of rapid growing rabbits compared with controls (Hernández *et al.*, 2005).

2.3. Phenotypic variations on carcass and meat quality at a fixed weight

The market weight for rabbits is fixed by market controls in each country. Then, the rapid-growing lines would be slaughtered at an earlier stage of maturity than non-selected animals, as a consequence of their higher adult weight. Again, there have been numerous experiments assessing the consequences of growth rate on quality traits at a fixed weight, by comparing breeds (Bernardini-Battaglini *et al.*, 1994; Dalle-Zotte and Ouhayoun, 1998) or lines selected for different objectives (Pla *et al.*, 1996; 1998).

On the other hand, very few experiments have investigated the consequences of a within-breed selection for growth rate (Gondret *et al.*, 2005). As expected when considering differences in physiological maturity, a lower carcass yield, reduced proportions of hind parts in the carcass, and a lower muscle:bone ratio have been reported between fast-growing and slow-growing rabbits (Gondret *et al.*, 2005). However, rapid-growing rabbits and controls shared many similarities for carcass composition in this experiment. Both divergently selected animals and controls displayed a similar fattening percentage, possibly because of an increase in appetite as a consequence of increasing growth rate. The lengths of femur and tibia bones were shortened with increasing growth rate, as a result of differences in the age of rabbits at slaughter. Interestingly, bone stiffness was reduced with increasing growth rate in the three lines (Gondret *et al.*, 2005).

Many similarities in meat quality traits were also found between rabbits with divergent growth rate slaughtered at a fixed weight. For instance, Gondret *et al.* (2005) did not find any changes in ultimate pH of the loin, WHC, and cooking loss between lines divergently selected on body weight at 63-days of age. Interestingly, ultimate pH in semitendinosus muscle was found lower in rapid-growing rabbits than in controls (Gondret *et al.*, 2005), but no changes in muscle metabolic enzymatic pathway at slaughter were found to explain such differences in meat pH.

Finally, there is no evidence for genetic differences in anti-oxidant enzyme activities in rabbit meat of different growth rates (Hernández *et al.*, 2002), which may indicate a similar behavior in selected and non-selected animals to lipid peroxidation through refrigerated storage.

2.4. Prospects for genetics of rabbit meat quality

The market for rabbit retail cuts is increasing in importance in European countries. Fore-, intermediate- (loin), and hind-parts of the carcasses obviously have different values. Therefore, selection of rabbits for an improved body composition may be interesting for the viability of the rabbit industry, following the example of chickens selected on breast yield (Berri *et al.*, 2001). The accuracy and cost of the selection process on carcass merit might be easier if ultrasonic scanning devices and (or) computerized image analysis were available to predict body composition and (or) meat quality in live animals.

Selection of growing rabbits of the Pannon White breed for both post-weaning ADG and cross-sectional area of the muscle longissimus has been made by using computerized tomography (Szendrö *et al.*, 2004 for a review). The value estimated for muscle surface was highly heritable ($h^2 = 0.41$). Rabbits with the highest muscle surface predicted in the longissimus muscle, also displayed a better dressing out percentage compared with non-selected rabbits. Altogether, the use of Pannon White rabbits selected on longissimus surface in terminal cross with Hyplus does seem to be advantageous for several traits related to carcass composition. For instance, cross-bred animals displayed a higher dressing out, an increased proportion of carcass intermediate part (loin), and a slightly improved muscle to bone ratio, and they did not show any degradation for meat pH and color (Metzger *et al.*, 2004). It remains to be determined whether selection for improved muscle cross-section area affected the muscle histological structure and final sensory traits of the meat. Obviously, the main limit of this

technique remains the access to a magnetic resonance imaging system.

Another concern might be the selection of rabbits for carcass fat content, since excessive fat deposition in the sold carcass is generally badly accepted by consumers. Rabbit carcass has a low fat percentage compared with other farm animals. However, due to the short generation interval in the rabbits and a given trend of lines selected by growth rate to become fatter, this trait should be reviewed from time to time in each selection program. Most attention has been paid in recent years to total body electro-conductivity measurement (ToBEC) to predict fat content in live animals without the need for chemical analyses. Milisits and Levai (2002) have shown the efficiency of divergent selection for ToBEC value on the carcass composition, with a difference of about 23% between rabbits selected for a low or a high fat content. However, Larzul and Rochambeau (2005) found that phenotypic correlation between ToBEC value and carcass adiposity estimated by weighing dissectible fat deposits, was approximately null in a commercial line of rabbits slaughtered at 65 days of age. It seems therefore that ToBEC would not work in lean young animals slaughtered at commercial weight, but is probably more accurate for adults, especially reproductive does. The method of scanning rabbits with a magnetic resonance tomograph also predicts the weight of perirenal fat with a very high accuracy (Köver *et al.*, 1996).

Finally, the heritability values of sensory traits are generally low in meat-producing mammals such as pigs (Sellier, 1998). Studies are therefore needed to evaluate the predictive values of muscle traits such as muscle fiber characteristics and meat fat content, because of their potential influence on sensory meat quality.

Altogether, rabbit genomic data are still limited. Until recently, only 39 loci have been identified (Fox *et al.*, 1993) and 55 genes have been precisely localized on the chromosomes (Zijlstra *et al.*, 2002; Hayes *et al.*, 2003). Only 160 full-length annotated rabbit genes are deposited in the GenBank database (Fadiel *et al.*, 2003). A genetic map is now under construction with micro-satellite markers every 10 to 20 centimorgans along the genome (Chantry-Darmon *et al.*, 2004). In the future, this frame will allow detection of linkage between a gene of interest and at least one of the markers, and therefore will permit marker-assisted selection in rabbits. Rabbit species might also take advantage of detection of chromosomal regions containing genes involved in performance (quantitative trait loci, QTL). However, considering the costs of a QTL scan, it is likely that the first QTL programs in rabbits will be restricted to disease resistance and reproductive traits. Several characteristics also make the rabbit an attractive model for transgenesis and experimental selection, including a short gestation period, a relatively high number of kits per litter, lifespan estimated around 9 years, a short interval between generations of selection, and a susceptibility to lipid-related disease close to humans. In addition, the rabbit genome has an almost equal size to that of humans. Therefore, many developments can be expected in the next years in the field of rabbit genomics.

3. Dietary effects on meat quality

The effects of nutrition on rabbit meat quality have been widely studied. The latest researches in dietary effects on meat quality have been focused on the use of different dietary fat sources to change fat content and composition, in order to produce meat with better nutritive value, sensory quality and (or) shelf life.

3.1. Effect of dietary fat on meat quality

The role of fat addition on rabbit growth performance and body composition has been reviewed by several authors (Maertens, 1998; Fernández *et al.*, 2000; Pla *et al.*, 2005). Increasing fat content of the diet generally results in a higher digestible energy (DE) intake, and both higher growth and feed efficiency. However, the carcass quality may be impaired with much greater adiposity (Pla and Cervera, 1997) when fat is present in the diet at very high levels (more then 9% of the ration).

It is well known that rabbits, as other non-ruminants, are able to incorporate dietary fatty acids into adipose and muscle tissue lipids. Short and medium chain fatty acids are mainly catabolized as energized sources in muscles, while the long chain fatty acids are mainly deposited directly in adipose tissues (Xiccato, 1998). Therefore, fatty acid composition is strongly affected by dietary lipid composition, but the fatty acid profile of rabbit meat and adipose tissues does not exactly reflect the fatty acid profile of the dietary fat source. This was probably due to the influence of exogenic fatty acids on in situ lipogenic enzyme activities (Gondret *et al.*, 1998c) and (or) elongation and desaturation processes in tissues. The effects of various dietary fat sources have been the subject of many experiments in the last years. The addition of vegetable fat compared to animal fat sources in the diet leads to differences in rabbit meat quality, especially regarding fatty acid composition of the tissues and meat flavour (Hernández *et al.*, 2000; Oliver *et al.*, 1997). For instance, sensory test panels attributed a higher “liver” taste to animals fed with an animal diet, while meats of animals fed with a vegetable diet had a higher “aniseed” or “grass” flavour. However, no differences between groups were found for the texture parameters evaluated.

The potential health benefits of ω3 essential polyunsaturated fatty acids (PUFA) in human nutrition (Connor, 2000) have stimulated an interest in increasing the n-3 PUFA level in meat and meat-

derived products. Besides, recommendations for human diets suggest decreasing the ω6:ω3 ratio. Different vegetable oil sources have been first used in rabbit diet to increase the level of lipid unsaturation (see Dalle Zotte, 2002 for a review). Recently, an attempt to specifically improve rabbit meat content in ω3 fatty acids has been carried out by Dal Bosco *et al.* (2004). These authors studied the synergistic effect of dietary alpha-linolenic acid as the main precursor of ω3 fatty acid family, and vitamin E as an anti-oxidant, on the oxidative stability and nutritional and eating characteristics of fresh and stored rabbit meat. This study confirmed the ability of rabbits to synthesise long chain PUFA (eicosapentaenoic and docosahexaenoic fatty acids) from the dietary precursor, leading to an increase in ω3 PUFA content of the meat of rabbits consuming the ω3 diet, without any alteration of oxidative stability and sensory quality of the meat. Other attempts have been made to determine the true impact of formulations with sources that are naturally rich in ω3 fatty acids, especially regarding the microbiological quality of rabbit meat. Vannini *et al.* (2003) showed that a dietary supplementation of whole linseed limited the growth rate of several microbial groups (except psychrotrophic bacteria), with a consequent increase in meat shelf life. In addition, dehydrated alfalfa meal at high percentages in the regimen seems to have also an inhibiting effect on microbial growth in rabbit meat products (Vannini *et al.*, 2002). Recently, some studies have also shed light on the possibility of manipulating ω3 PUFA content in rabbit carcasses during the early growth period (Castellini *et al.*, 2005; Muñiz *et al.*, 2005). Although primarily devoted to improving the immune status of the young rabbits, these two studies demonstrated that dietary maternal ω3 PUFA are secreted in the milk, and then allow a ω3 enrichment in the young tissues. Adequate distribution periods of ω3 enriched-diets remain to be determined in rabbits.

Conjugated linoleic acid (CLA) has also received a great deal of attention as a supplement in rabbit feed that can favourably modify body composition (Corino *et al.* 2002; 2003), due to its potential to increase lean tissue deposition while decreasing fat deposition in various species (Dunshea *et al.*, 2005). Besides, it has potential nutritional benefits for Human, because it has anti-obesity (Lin *et al.*, 1995) and anticarcinogenic activities (Ip *et al.*, 1996), it is able to ameliorate diabetes (Housseknecht at al., 1998), and it has a protective effect against atherosclerosis at least in rabbits (Lee *et al.*, 1994). Rabbit growth performance and carcass characteristics at standard slaughter weight (2.5 kg, 76 d) were not affected by conventional pelleted diets supplemented with 0, 0.25, or 0.5% of a CLA preparation. However, CLA supplementation reduced perirenal fat weight at heavy slaughter weight (3.1 kg), and lowered concentration of serum triglycerides and total cholesterol (Corino *et al.* 2002). Regarding the chemical composition of rabbit meat, a significant decrease in meat lipid content was evidenced only when rabbits fed diet with a high supplementation level of CLA (0.5%) were considered at heavy slaughter weight (3.1 kg; Corino *et al.* 2003). Finally, CLA supplementation may have positive effects on meat texture and tenderness, since the intramuscular collagen and hydroxylpyridinoline crosslinks were lower in intermediate levels of dietary CLA supplementation. However, sensory properties of the meat remain to be directly investigated.

3.2. Antioxidants

Lipid oxidation is a major nonmicrobial factor responsible for the quality deterioration of muscle foods. It leads to discoloration, higher drip-loss, and the development of off-odour and off-flavor (Monahan 2000). The dietary manipulation of tissue lipid composition to produce meat with a high content of PUFA could reduce the oxidative stability of the meat-products and have a negative effect on meat quality. For instance, dietary inclusion of 8% of whole linseed increased lipid peroxidation in rabbit meat as evaluated by induced thiobarbituric acid-reactive substances (TBARS), leading to impaired sensory properties of rabbit meat during storage (Bianchi *et al.*, 2003). For this reason, there has been an increasing interest in recent years in the use of antioxidants in rabbit formula.

Vitamin E is commonly used in animal feed, as an indispensable component of biological membranes with stabilizing properties and a high antioxidant activity. Vitamin E is the generic term used to describe at least eight naturally occurring compounds that exhibit the biological activity of α-tocopherol (Morrissey *et al.*, 2000). In recent years, different studies have been carried out in rabbits to study the effects of dietary extra supplementation with vitamin E on the deposition of α-tocopherol in tissues, on meat quality characteristics, and on oxidative stability and the shelf life of the meat. Several authors (Castellini *et al.* 1999, López-Bote *et al.* 1997) have shown that the deposition of α-tocopherol in rabbit muscle is very efficient and has a strong relationship with the supplementation level used in the diet. Dietary α-tocopheryl acetate supplementation has been found to stabilise colour of raw meat (Corino *et al.*, 1999), even after refrigerated storage (Dalle Zotte *et al.*, 2000). Vitamin E has been also effective in reducing lipid oxidation during refrigerated and frozen storage of meat (Castellini *et al.* 1999; Lo Fiego *et al.*, 2004). In addition, vitamin E supplementation increases the oxidative stability of cooked rabbit meat (Castellini *et al.* 1999), whatever the different cooking methods studied (Dal Bosco *et al.* 2001). Besides, a high α-tocopherol level improves some physical traits of

meat, reducing shear values and increasing water holding capacity (Castellini *et al.*, 1998). The effect of dietary synergetic supplementation of vitamins C and E have been also investigated, leading to an increase in the vitamin content and reducing the oxidation of the lipids (Castellini *et al.* 2000; Lo Fiego *et al.*, 2004).

Different natural ways of improving the oxidative stability of rabbit meat have also been studied. For instance, rabbit lipid oxidative stability was improved by increasing the level of oats in rabbit diet (López-Bote *et al.*, 1998). Coni *et al.* (2000) also have verified the antioxidant efficiency of extra virgin olive oil and oleuropein, an olive oil biophenol, in rabbit plasma and isolated low density lipoproteins (LDL). However, it seems that oleuropein did not reduce meat susceptibility to oxidation (Paci *et al.*, 2001).

3.3. Effect of feed restriction on rabbit meat quality

For several species, some production systems proposed to improve meat sensory quality are based on increasing the age at slaughter, in order to produce more mature animals and to improve sensory meat quality. Feed restriction systems could be then useful for some niche productions systems, for which a minimum age at a given slaughter weight is generally imposed. Recently, it has been also established that quantitative feed restriction could have a favourable impact on post-weaning enteropathy (Gidenne *et al.*, 2003). In rabbits, feed restriction generally decreases growth rate and has a detrimental effect on feed conversion and both carcass and meat quality (see Xiccato, 1999, for a review). Better growth performance is generally obtained with early feed rationing followed by feed intake close to *ad libitum* level, than with late feed rationing (Dalle Zotte *et al.*, 2005b). The carcasses of restricted animals generally showed lower dressing percentage, fat percentage, and muscle proportion (Gondret *et al.*, 2000; Larzul *et al.*, 2004). Besides, the meat showed higher water content and lower lipid content, probably due to a decrease in the activities of the enzymes implicated in fatty acid biosynthesis (malic enzyme and glucose-6-phosphate dehydrogenase; Gondret *et al.*, 1997). However, sensory properties at least in *orylag*® genotypes seemed to be unaffected by feed restriction (Larzul *et al.*, 2004).

Several studies in other species (Seideman and Crouse, 1986, in beef; Solomon and Lynch, 1988, in lambs; Solomon *et al.*, 1988, in pigs) have pointed out that restricted feeding increases the percentage of oxidative fibres and favours the oxidative metabolism pathway. However, no such modification of muscle fibre characteristics was observed in rabbits (Gondret *et al.*, 2000; Dalle Zotte *et al.*, 2005b). Likewise, the activity level of the isocitrate dehydrogenase, i.e. an enzyme implicated in the oxidative energy metabolism, was not influenced by feed rationing (Dalle Zotte *et al.*, 2005b). Recent development in rabbit researches has been also conducted on the long-term effects of early feed rationing. A delayed maturation of the muscle fibres of new-born rabbits as a consequence of early pre-natal restriction has been evidenced at weaning (Gondret *et al.*, 1997). However, no effect of maternal feed rationing (80% of *ad libitum* intake) during gestation was found on muscle fibre size and types in the offspring at commercial slaughter (Dalle Zotte *et al.*, 2005a).

3.4. New trends in the interactions between meat quality and nutrition

Most of the researches conducted in recent years, and to be carried out in the future, have focused on incorporating bioactive compounds in meat for the benefit of human health.Specifically, rabbit meat could become a good way of providing these bioactive compounds to human consumers, since the manipulation of rabbit diet is very effective in increasing the levels of ω3 PUFA, CLA, or vitamin E. In addition, both selenium and iron are also responsive to dietary supplementation in rabbits (Lynch and Kerry, 2000).

A new tool to improve meat quality is nutrigenomics, a science that tries to combine genetics and nutrition. Nutrigenomics aims at studying the effects of dietary bioactive compounds on gene expression (Hirsch and Evans, 2005, Müller and Kersten, 2003). This science could have an impact in preventing and even treating diseases through targeted nutrition (Kauwell, 2005). The potential of nutrigenomics as an emerging strategy in modern meat science has been recently discussed (Andersen *et al.*, 2005). These authors pointed out that nutrigenomics will have significant implications on future strategies to control meat qualities. The ongoing mapping of the genomes of the principal farm animals, including rabbits, and the progress in molecular biological techniques will probably accelerate the progress of the nutrigenomics. However, considering the costs of these studies, it seems that it will be difficult to apply these new technologies nowadays to rabbit meat quality.

4. Influence of types of rearing on rabbit meat quality

Conventional breeding systems for weaned rabbits generally involve bi-cellular or collective wire-cages. Cages are disposed in indoor buildings either on a flat-deck or in a semi-Californian arrangement. Intensive systems then allow the production of rabbits at the commercial weight in approximately 60-80 days after birth, depending on the breed and slaughter weight specific to each country. However, the rabbit meat industry should take into account new consumer needs in terms of

meat quality, including both real and perceived attributes of the products. In addition to sensory properties, animal welfare, environmental impact and cultural aspects of the production systems are now important components of meat quality. Therefore, niche markets for rabbits that require production with specific attributes (age at slaughter, type of housing, stocking density, organic feeds …) are being developed. The number of alternative production systems in European Union remains low, but many researches have been carried out on these systems in recent years. Changes from confinement to caged-systems including a different form of space organization (platform, tunnel) or to more-free range systems have been first the objectives of many researches for rabbit does (Mirabito, 2003; for a review). Other attempts including large indoor pens or totally open-air systems have now been tested also for growing rabbits, (Combes and Lebas, 2003 for a review). Many interacting components are involved in the outcome of alternative rearing systems compared with conventional ones, such as weight or age at slaughter, genetics, nutrition, physical exercise, stocking density, type of floor, environmental temperature, and health status. Therefore, it is difficult to compare carcass merit and meat quality from one system to another, and to assess the precise factors underlying the observed variations between alternative systems and conventional housing.

4.1. Main limitations associated to conventional rearing systems

Rabbit breeding in conventional wire cages presents a number of problems with respect to animal protection legislation (Stauffacher, 1992). Indeed, traditional wire floor may cause leg and footpad injuries (Drescher, 1992; Rommers and Meijerhof, 1996), that may be solved by the general use of plastic floors. It is thought to offer limited sources of distraction and stimulation to animals, even if investigations revealed that rabbits generally prefer wired floor to dirty straw bedding (Morisse *et al.*, 1999). Furthermore, conventional breeding system is perceived by consumers as far from the natural behaviour and social needs of rabbits. Indeed, conventional cages do not allow animals to hop and stand up, and greatly limit locomotion. Single- and double-housing systems obviously reduce social contacts (Drescher, 1992). On the opposite, collective cage housing in a limited space generally causes social conflicts if extended more than 12 weeks, due to aggressive behaviour after puberty. Finally, the widespread use of sub-therapeutic antibiotics in intensive breeding systems is detrimental for the image of the products.

Conversely, consumers generally share a positive attitude towards outdoor-reared animals and organic production, since they perceive animal welfare to be enhanced, smell and pollution to be reduced, and meat to be safer with lower use of feed additives and antibiotics compared with conventional production. Indubitably, pen-reared rabbits show a more active behaviour than rabbits reared in conventional cages, spending more time on movement, social activity and comfort than on resting and ingesting (Dal Bosco *et al.*, 2002). Numbers of jumps, runs, and stand up positions are then clearly increased with increasing space allowance (Postollec *et al.*, 2003). The presence of hay or grass cubes in pens generally decreases stereotype behaviour. However, results on animal welfare are less clear when straw litter is used instead of wire-netting, because of sanitary problems associated with deep bedding (Mirabito, 2003; Combes and Lebas, 2003 for reviews). Finally, the introduction of wood sticks or toys in wire-netting housing appears to restore the chewing behaviour, which may be considered as a benefit for rabbit welfare. Results must be however interpreted with caution regarding definition of “abnormal” behaviour, feeding condition, and form of presentation of the enrichment in the pens.

4.2. Carcass merit and meat quality in indoor pen-housing systems

There has been considerable work comparing post-weaning performance of rabbits housed in pens to animals reared in conventional cages. Rabbits reared in pens (0.6 to 10.2 m²) generally showed deteriorated performance, with reduced feed intakes and lower ADG compared with cage-reared rabbits (Maertens and Van Herck, 2000; Maertens and Van Oeckel, 2001; Dal Bosco *et al.*, 2002; Jehl *et al.*, 2003; Postollec *et al.*, 2003; Metzger *et al.*, 2003). Presumably, growth requirements were not adequately met by voluntary ingestion in alternative systems, due to the time spent in activities other than feeding. However, Postollec *et al.* (2003) did not show any variation in feeding behaviour in relation to space area. Therefore, the alteration observed by some authors (Dal Bosco *et al.*, 2002) in gain:feed ratio may be considered as a response to variation in stocking density rather than to housing system itself (Maertens and Van Oeckel, 2001; Postollec *et al.*, 2003).

The reduction of growth rate in pen-raised rabbits obviously affected slaughter weights at the same age, and thus carcass merit and meat traits. Generally, dressing out percentage was unaffected by the housing systems (Maertens and Van Oeckel, 2001; Combes *et al.*, 2003c; Jehl *et al.*, 2003). Some studies observed a slight reduction in carcass yield for pen-raised rabbits compared with those held in cages (Lambertini *et al.*, 2001; Dal Bosco *et al.*, 2002), probably due to a confusing influence with stocking density in the different systems (Xicatto *et al.*, 1999). On the other hand, carcasses of rabbits reared in large indoor pens consistently had a higher proportion of hind parts and thigh compared with

those of cage-reared animals (Dal Bosco *et al.*, 2000; Combes *et al.*, 2003c; Jehl *et al.*, 2003; Metzger *et al.*, 2003). Since rabbits in both systems were generally slaughtered at the same age in these different experiments, the modification observed in the proportion of retail cuts for pen-housed rabbits may be due to difference in maturity degree (weight/adult weight) compared to animals held in cages, but also may be considered to a specific response to exercise.

In support of the latter assumption, forced jump exercise in giant cages equipped with hurdles also increased the proportion of hind-parts in the carcass, when exercised rabbits were compared to confined animals at a same age and similar body weight (Combes *et al.*, 2004). Besides, bone weight and stiffness were increased for rabbits reared in pens compared with cage-housing systems (Jehl *et al.*, 2003). However, authors reported either a lower (Jehl *et al.*, 2003) or the same ratio of meat to bone (Dal Bosco *et al.*, 2002; Combes *et al.*, 2003c) in carcasses of animals reared in pens compared with those held in wire conventional cages. Raw meat texture remained unaffected by housing systems, but cooking losses tended to be higher with expanded space allowance (Combes *et al.*, 2003c).

Another major effect of pen housing was the reduction of carcass fat depots compared with conventional cages (Dal Bosco *et al.*, 2002; Combes *et al.*, 2003c; Jehl *et al.*, 2003; Metzger *et al.*, 2003), although Maertens and Van Oeckel (2001) observed a similar adiposity between the different housing systems. Combes *et al.* (2004) did not observe any variation in carcass fatness between exercised rabbits in giant cages with hurdles and confined animals. The variation of fat proportion found in different studies dealing with pen housing systems may be then related to factors (stocking density, appetite, …) other than physical exercise. Muscle lipid and meat fat contents generally followed the same pattern than carcass fatness (Lambertini *et al.*, 2001; Metzger *et al.*, 2003). Furthermore, pen housing systems led to clear modifications in fatty acid profile, with a decrease in the proportion of monounsaturated fatty acids and a higher proportion of polyunsaturated fatty acids in the longissimus muscle (Dal Bosco *et al.*, 2002) in pen-housed rabbits compared with conventional animals.

Glycogen content is another important factor in the upset of meat quality, since glycogen stores at slaughter partly condition the rate and amplitude of pH fall during the post-mortem period. Glycogen concentration in skeletal muscles was found higher for pen-raised rabbits than for confined caged-animals (Dal Bosco *et al.*, 2001). The explanation probably lay in the difference between animals in stress susceptibility during transport and the handling process. Indeed, although a better ability of skeletal muscles to oxidize fatty acids has been shown in rabbits in response to forced exercise during growth, this was not associated with any variation in glycolytic potential (i.e., an estimator of glycogen content *in vivo*) and ultimate pH in exercised rabbits compared with confined ones (Gondret *et al.*, 2004). Finally, variations observed in ultimate pH of the meat between housing systems were rather controversial, with reports of a lower meat pH in pen-raised rabbits than cage-reared animals (Dal Bosco *et al.*, 2001; 2002; Lambertini *et al.*, 2001), or lack of changes (Combes *et al.*, 2003c; Jehl *et al.*, 2003). Pen housing resulted in either a higher (Dal Bosco *et al.*, 2002; Combes *et al.*, 2003c), the same (Combes *et al.*, 2003c; Jehl *et al.*, 2003) or a lower meat lightness (Maertens and Van Oeckel, 2001), depending of experiments and muscles. Also, heterogeneous results were found for other colour traits in response to housing systems (Dal Bosco *et al.*, 2001, 2002; Maertens and Van Oeckel, 2001; Combes *et al.*, 2003c).

Combes and Lebas (2003) evidenced a threshold for stocking density, above which increasing the number of rabbits per m² would greatly depress individual growth performance. However, carcass yield, hind part proportion, and muscle to bone ratio seemed to be relatively independent of stocking density in the pens. Reviewing different experiments, the same authors concluded that stocking density alone did not, or only slightly affected, meat quality traits.

Indoor pen housing systems then resulted in modifications of growth performance and carcass composition when compared with conventional wire cages in rabbits at the same age. However, even when associated with modifications in type of feeding and breed to meet "Label-Rouge" specifications, indoor pen-housing system did not affect sensory meat quality compared with conventional caged systems (Jehl and Juin, 2001).

4.3. The specific influence of environment enrichment in indoor pens

Bedding (usually straw) is an important environmental factor. In addition to absorbing urine and feces, the straw is used by rabbits for playing. However, the consumption of large amounts of straw generally reduced total feed intake and slightly increased the mortality for rabbits reared in straw-bedding pens compared with those held in wire-net pens (Dal Bosco *et al.*, 2002). Even when wire-pens were equipped with a straw hopper, a decrease in feed consumption resulting of straw ingestion was noted in comparison with conventional net-cages (Maertens and Van Oeckel, 2001). Then, performance was generally worse in straw-litter pens than in wire-net pens or cages, with reduced ADG, lower dressing out, and altered meat-to-bone ratio (Dal Bosco *et al.*, 2002; Metzger *et al.*, 2003).

Table 7. Summary of the main variations observed in growth performance, carcass composition and meat quality traits for rabbits reared in pens in or ou-of-doors, compared with rabbits held in conventional wire cages[1].

Traits	Indoor pens	Outdoor housing
Average daily gain	↘	↘
Carcass yield	↘ or =	↘ or =
Hind part proportion	↗	↗
Meat:bone ratio	↘ or =	=
Carcass adiposity	↘ or =	↘
Meat fat content	↘ or =	↘
Polyunsaturated fatty acids percentage	↗	↗
Ultimate pH	↘ or =	↘
Cooking loss	↗	
Sensory traits	=	=

[1]References are given in the text

Presence of a platform to organize space in cages, or addition of wood sticks or a straw hopper to improve rabbit welfare, has also been tested. Performance and product quality were rather similar for rabbits reared in pens with or without enrichment (Maertens and Van Oeckel, 2001; Luzi *et al.*, 2003; Postollec *et al.*, 2003). The presence of a platform alone in wire cages did not lead to marked modifications in carcass and meat quality (Postollec *et al.*, 2003; Combes *et al.*, 2003c), although it was able to modify hind-part proportion in the carcasses (Jehl *et al.*, 2003). Furthermore, specific improvements of the enrichment system are still needed to avoid excreta contaminations of rabbits located below the platform.

4.4. Carcass merit and meat quality in outdoor production systems

Outdoor rabbit production is defined as a system that allows outside access during whole or a part of the growing period, including or not contact with pasture and soil. Another major difference between indoor and outdoor systems is the exposure to fresh air and greater extremes of climate. Both flat-deck outdoor collective cages below a roof (Margarit *et al.*, 1999; Luzi *et al.*, 2000) and wooden multi-tier cages (Fijal *et al.*, 2000) have been developed (total open-air system). Also, large indoor pens with access to an outdoor plot (partial outdoor systems) have been compared with conventional housing system totally in barn (Van der Horst *et al.*, 1999).

The main result was the reduction of ADG for rabbits totally reared or only finished out of doors, compared with indoor caged-rabbits (Table 7; Margarit *et al.*, 1999; Van der Horst, 1999; Luzi *et al.*, 2000; McNitt *et al.*, 2003). This was also associated with a degradation of the feed conversion ratio (Van der Horst *et al.*, 1999; Fijal *et al.*, 2000).

Dressing out was depressed (Van der Horst *et al.*, 1999) or not (Luzi *et al.*, 2000) for outdoor rabbits compared to conventional animals. However, meat:bone ratio did not seem to be affected by outdoor housing system (Margarit *et al.*, 1999). Similar to modifications observed in indoor pens (Table 7), the proportion of hind-parts in the chilled carcasses was higher (Luzi *et al.*, 2000), fat proportion was lower, and muscle fat content was decreased in rabbits reared totally or partially out of doors compared to conventional indoor animals (Margarit *et al.*, 1999; Van der Horst *et al.*, 1999; Cavani *et al.*, 2000). Conversely, a higher content of polyunsaturated fatty acids was also reported in open-air reared rabbits with access to a fresh pasture, compared to conventionally-reared animals (Cavani *et al.*, 2004).

Ultimate pH was lower in meat from outdoor-reared rabbits compared with that from indoor caged animals (Cavani *et al.*, 2000). However, this did not result in difference in WHC of the meat and cooking loss among production systems (Cavani *et al.*, 2000).

Finally, pen-housing systems out of doors did not affect rabbit sensory meat quality compared with

conventional caged housing systems (Margarit *et al.*, 1999; Cavani *et al.*, 2004).

4.5. Carcass merit and meat quality in organic production

A small number of farms at present produce organic rabbits. Standards for organic rabbit production are in accordance with European Community standards for organic livestock and livestock products (Council Regulation EC 1804/1999 amending Directive EEC 2092/91), with additional specifications sometimes being imposed by individual countries. Age at slaughter is fixed to a minimum of 100 days for organic rabbits. Obviously, diets have to respect an organic definition, especially regarding the lack of anti-coccidian supplementation, and a farm origin for the majority of the feedstuff. Fresh, dry or lyophilised forages must comprise at least 60% of the dietary dry matter intake. Breeding animals generally have access to pasture, however a concrete floor is accepted with organic straw or wood chip litter. Only wire net is forbidden (Marionnet, 2000). Animals must have an access to open-air, but partial indoor housing is permitted. Although not compulsory, emphasis is generally placed on traditional breeds adapted to local conditions.

Both fenced fields (200 m²-400 m²; Bradley and Hague, 1996) and moving giant cages without base netting (2.8 m²; Lebas *et al.*, 2002; Combes *et al.*, 2003a) have been compared with conventional cages. Few studies dealing on carcass meat quality traits are however available. As reported for pen housing systems, organic production led to a reduction in ADG (Salcedo-Bacca *et al.*, 2004), probably because feeds are not optimized for growth requirements. Combes and colleagues (2003a, b) have shown that organic rabbits had a better carcass yield than conventional rabbits slaughtered at a same weight, and they also displayed higher hind-part proportions, a lower interscapular fat percentage, and a reduced muscle fat content. In addition, raw muscles had a higher mechanical firmness when obtained from organic rabbits compared with conventional animals. Mechanical tests performed on cooked samples were rather controversial among muscles (Combes *et al.*, 2003b). Ultimate pH of the meat was higher and meat colour was darker in organic production compared with conventional system (Combes *et al.*, 2003a).

The loin meat from organic production was clearly distinguished from that of standard production by a trained sensory panel: it obtained a higher score for tenderness (Combes *et al.*, 2003b), although organic raw meat showed a higher mechanical firmness. Other sensory traits under study (juiciness, flavour) were not modified by production systems. Since organic rabbits are given access to pasture or fresh forage, they theoretically have higher levels of polyunsaturated fatty acids of n-3 family. In agreement, Pla *et al.* (2006) found that meat of organic produced rabbits was poorer in monounsaturated fatty acids and richer in polyunsaturated fatty acids than that from conventional production. Direct nutritional effect of herbage could be however accompanied by indirect negative effects on total nutrient intake. Therefore, more assessments of nutritional values of rabbit meat from organic production are needed.

4.6. Conclusions

Many rearing systems have now been developed for growing rabbits as an alternative to the conventional wire-cages, including an increased space area allowance, a higher number of animals to interact, and eventually specific environmental enrichments. Both indoor and outdoor pen-housing systems generally resulted in a reduction of growth performance. Despite a better formation of carcasses with an increase in the proportion of hind-parts and less fat in rabbits reared in pen housing systems, meat quality did not seem to be greatly modified compared with conventional systems. Furthermore, most of the differences observed in carcass merit and meat traits were probably due to the difference in maturity for rabbits slaughtered at the same age. Finally, numerous problems in health and sanitary concerns made it difficult to recommend alternative housing systems other than for niche productions. Apart from organic production, it therefore seems difficult to claim alternatively-reared rabbits of superior objective quality. Whether consumers are willing to pay more for rabbit with attributes linked to animal welfare remains to be demonstrated.

5. Microbiological quality of rabbit meat

Information on rabbit microbiological quality is scarce (Bard, 2004; Berruga *et al.*, 2005; Cabanes *et al.*, 1994; Khalafalla, 1993; Pérez-Chabela *et al.*, 1999 ; Rodríguez-Calleja *et al.*, 2004, 2005a,b). Rodríguez-Calleja *et al.* (2004) studied the microbiological quality of 24 h post-mortem chilled rabbit carcasses and prepackaged rabbit meat stored chilled in air for 0 to 3 days at the retail level. The mean total bacteria count was between 4.01 - 4.96 log cfu/g depending of the abattoir. The dominant contaminants on carcasses and prepacked rabbit meat were *Pseudomonas*, lactic acid bacteria, yeasts and *Brochothrix thermosphacta.*

The microbiology of carcasses is greatly dependent on the conditions under which animals are reared, slaughtered and processed. Thus the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature, and other conditions of storage and distribution are the most important factors determining meat microbiological quality (Brown and Baird-Parker, 1982). Unless effectively controlled, the slaughtering process may cause

extensive contamination of the muscle tissue with a vast range of microorganisms. Some of these microorganisms come from the animal intestinal tract and others from the environment in contact with the animals before or during slaughter. López *et al.* (2002) have studied the evolution of most important contaminant and pathogen biota on carcasses during the different steps of rabbit slaughter process. Figure 1 shows the main results of this study. Total counts of aerobic and mesophilic microorganisms increased during the slaughtered process but were slightly reduced after chilling. Mean counts were 4.17 log cfu/g in skinned carcasses; increased until 5.89 log cfu/g during evisceration and dressing, and showed a reduction to around 4 log cfu/g after chilling process. In the same way, counts of yeasts and moulds increased during dressing process, showing final counts of 3.59 log cfu/g. *Enterococcus spp.* were not present initially, but their number rose to 4.58 log cfu/g after evisceration; however, after chilling their counts were significantly reduced (0.78 log cfu/g). *Enterobacteriaceae* counts were very low in skinned and chilling carcasses, reached a number of 5.17 log cfu/g after the evisceration, and were finally reduced to nearly 1 log cfu/g during the chilling process. Similarly, coliforms were present after evisceration (5.37 log cfu/g), but in skinned and chilling carcasses these microorganisms were not present. Besides, *Escherichia coli* was not present in skinned carcasses but the evisceration and posterior processes increased its number. The chilling process reduced *E. coli* counts but did not eliminate totally its presence. *Escherichia coli* and also *Enterobacteriaceae* and coliforms have an enteric origin, so evisceration process had a determinant influence in their counts. *Clostridium perfringens* appeared on the carcasses during evisceration, and its number rose after chilling (1.20 log cfu/g). *Listeria monocytogenes*, *Salmonella spp.* and *Campylobacter spp.* were not found in any step of the process.

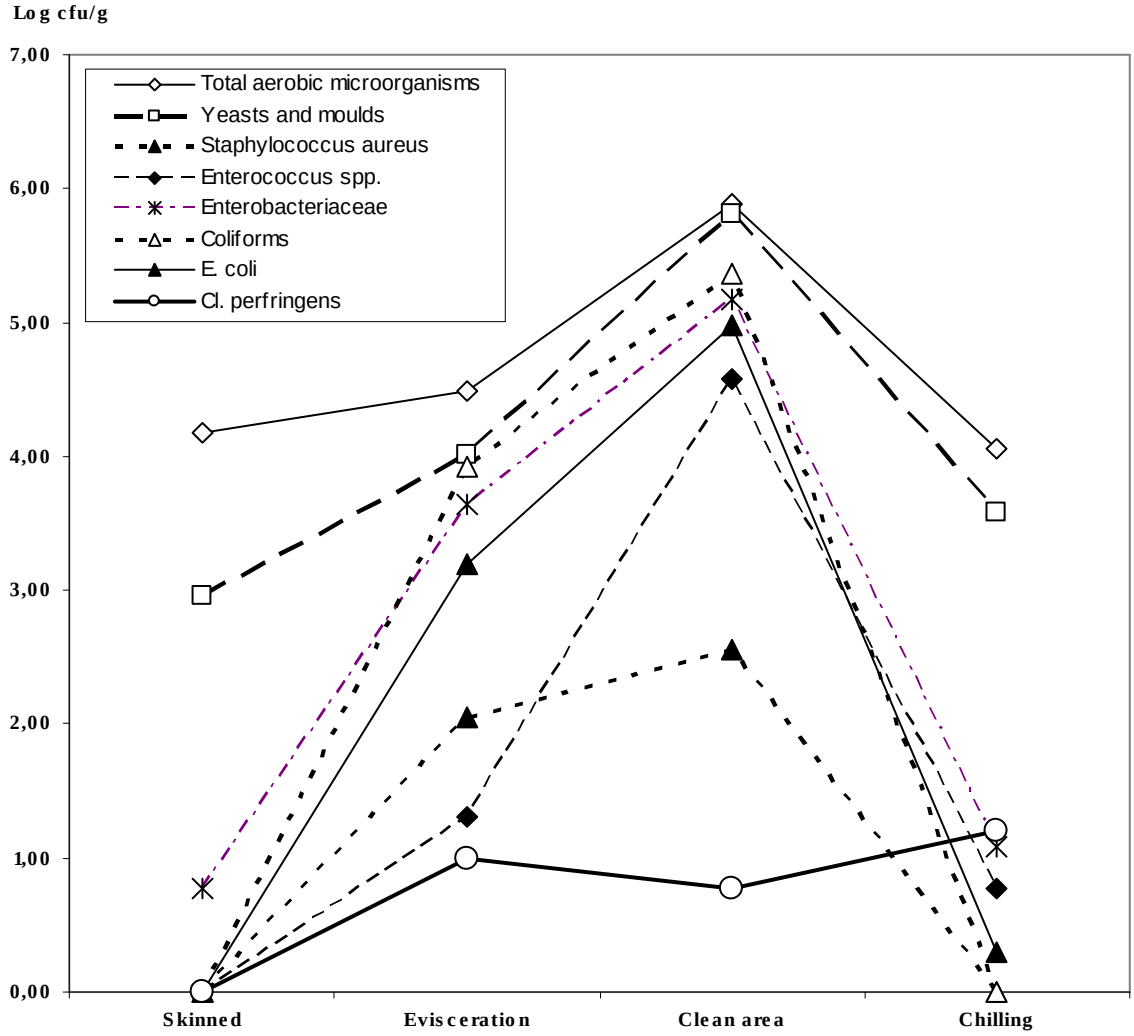

Figure 1. *Evolution of microbial counts during rabbit slaughter process.*

Microbial flora is a limiting factor that determines the shelf life and the safety of meat. It has been established that microbial levels of 6-7 log cfu/g are critical for the spoilage of meat. Rodríguez-Calleja *et al.* (2005a) studied the shelf life of rabbit carcasses, overwrapped with oxygen-permeable film and stored at 3 ± 1ºC during 8 days. For both, appearance and odour, the average shelf life of rabbit carcasses was estimated to be 6.8 days when mean of aerobic plate counts were ca. 8 log cfu/g. Nevertheless, the counts of these bacteria were ca. 7 log cfu/g after 5 days of storage and most of the carcasses already showed some softening. All carcasses exhibited a visible slime layer after 8 days of storage. However, Bobbitt (2002) estimated a shelf life of rabbit carcasses of three days at 4ºC. The differences in estimation of the shelf life could be explained by differences in initial microbial counts, since a high initial contamination of meat reduces product shelf life (Gil *et al.*, 1998). Recently, some attempts have been made to increase the shelf life of rabbit meat using modified atmospheres (Berruga *et al.*, 2005) or irradiation (Badr, 2004; Rodríguez-Calleja *et al.* 2005b).

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5.2. Rabbit meat traceability

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1. European legislation

European rabbit production has been influenced by the introduction of more restrictive regulations and higher consumer attention to food safety aspects (Verbeke, 2001; Schwägele, 2005). The European Union has enacted several regulations aimed at guaranteeing meat safety and systems to prevent or at least manage similar future crises (Table. 1). The major objective was to enforce the provision of clear and reliable information to consumers at sale points, based on a system of tracking meat back to the animal of origin, the slaughterhouse and the cutting unit. In the EU since January 1st 2005 (Regulation 178/2002/EC), it has been compulsory for all feed and food operators to adopt a traceability system. They have been able:

"to identify any person from whom they have been supplied with a food, a feed, a food-producing animal, or any substance intended to be, or expected to be, incorporated into a food or feed. To this end, such operators shall have in place systems and procedures which allow for this information to be made available to the competent authorities on demand" (Article 18).

Before this, a Concerted Effort Framework – 'FoodTracE' was established by the EU in 2000 aimed at developing a practical framework for traceability of food and developing the means to plan, model, validate and implement the traceability process. Moreover, many of the large food manufacturers, retailers and food service companies have already established traceability arrangements, primarily to reduce business risk (Furness and Osman, 2003). In addition to a EU proposed regulation, several countries introduced their own regulations on traceability.

Furthermore, an International Standards Organisation (ISO) definition is also to be found that defines traceability as "the ability to trace the history, application or location of an entity by means of recorded information (ISO 8402:1994). The ISO definition, whilst more general in terms of the traceability entity, draws attention to the importance of recorded information that is essential for satisfying traceability requirements.

While the EU and the U.S. seem to adopt different approaches, in practical terms the European and the American positions on food safety/security and traceability are remarkably similar: a mandatory requirement on operators to maintain records for 'product tracing', sectoral requirements enforceable by inspection and extension of traceability to other attributes of interest to processors/retailers/ consumers including composition and processing (Smith *et al.*, 2005).

2. Key elements in a traceability system

A company should always use legislative, trade association or accepted industry practice as the standard for setting up and verifying a traceability management system (Dillon and Thompson, 2003). In particular:

- the business must have established procedures to trace all raw materials of a finished product by lot marking or batch code, and to identify the location of packaged product during distribution, to allow withdrawal or recall of any product retrospectively found to be out of specification.

- the business must ensure that a recall system, detailing key personnel, out of hours contacts and responsibilities for decision making, is fully documented.

- the business must test the traceability system to prove that relevant controls work, and also test yearly the product recall mechanism to ensure control remains effective.

The regulation requires traceability at all stages of the supply chain, from the feed given to animals to the consumption of the final product by the consumer, so it integrates traceability into the

implantation of wider food safety management systems based on the principles of Good Manufacturing Practice (GMP), Hazard Analysis in Critical Control Points (HACCP) and ISO 9000 (Fig. 1) (Zandernowski *et al.*, 2002).

GMP is now considered as a prerequisite for safe food production and has been used for many years to ensure the microbiological and chemical safety and quality of food. The establishment of GMP is the outcome of long practical experience and attention to environmental conditions in the farm and slaughter/processing plant, e.g., requirements for farm/plant layout, hygienic design of equipment and control of operational procedures. However, the GMP concept is largely subjective and its benefits are only qualitative. Also it has no direct relationship with the safety status of the product. For these reasons, the concept has been extended by introducing the HACCP system. The HACCP concept is a systematic approach to the identification, assessment and control of hazards in a particular production operation. It aims to identify problems before they occur and establish measures for their control at stages in production that are critical to ensuring the safety of the food. The role of GMP is to ensure that hygienic equipment is used, that well-trained personnel are involved in the production process, that re-contamination is avoided, etc. HACCP is the managerial tool in ensuring that the chosen criteria are met. Additional compliance with ISO 9000 ensures that the products will be of high repeatable quality (Zandernowski *et al.*, 2002; Dillon and Thompson, 2003).

Table 1. European Union legislation in force on traceability and food labelling.

SOURCE	ACTS OR REGULATIONS
European Commission 12 January 2000	**White paper on food safety** Introduction of the concept of traceability and the establishment of a European Food Authority.
European Parliament and Council 17 July 2000	**EC Regulation 1760/2000** Establishing a system for the identification and registration of bovine animals and regarding the labelling of beef and beef products.
European Parliament and Council 12 March 2001	**EC Directive 18/2001** Release of genetically modified organisms was adopted by the European Parliament and the Council of Ministers in February 2001 and entered into force on 17 October 2002.
European Commission 25 July 2001	**Regulation proposal EC of 25/07/2001 2002/C 125/14** Opinion of the Economic and Social Committee on the "Proposal for a Regulation of the European Parliament and of the Council concerning traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC".
European Parliament and Council 28 January 2002	**EC Regulation 178/2002** Definition of traceability as the ability to trace and follow food, feed, and ingredients through all stages of production, processing and distribution.
European Parliament and Council 22 September 2003	**EC Regulation 1830/2003** Concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms.
European Parliament and Council 29 April 2004	**EC Regulation 854/2004** This regulation supplements the regulations on hygiene of foodstuffs, on specific hygiene rules for foodstuffs of animal origin and on official controls on food and feed.

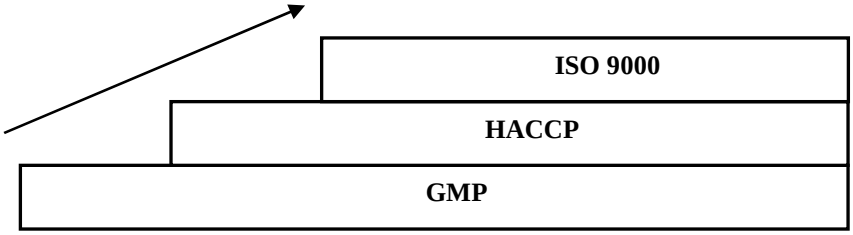

Figure 1. *General quality chain: phases and order of food safety system implementation (adapted from Zandernowski et al., 2002).*

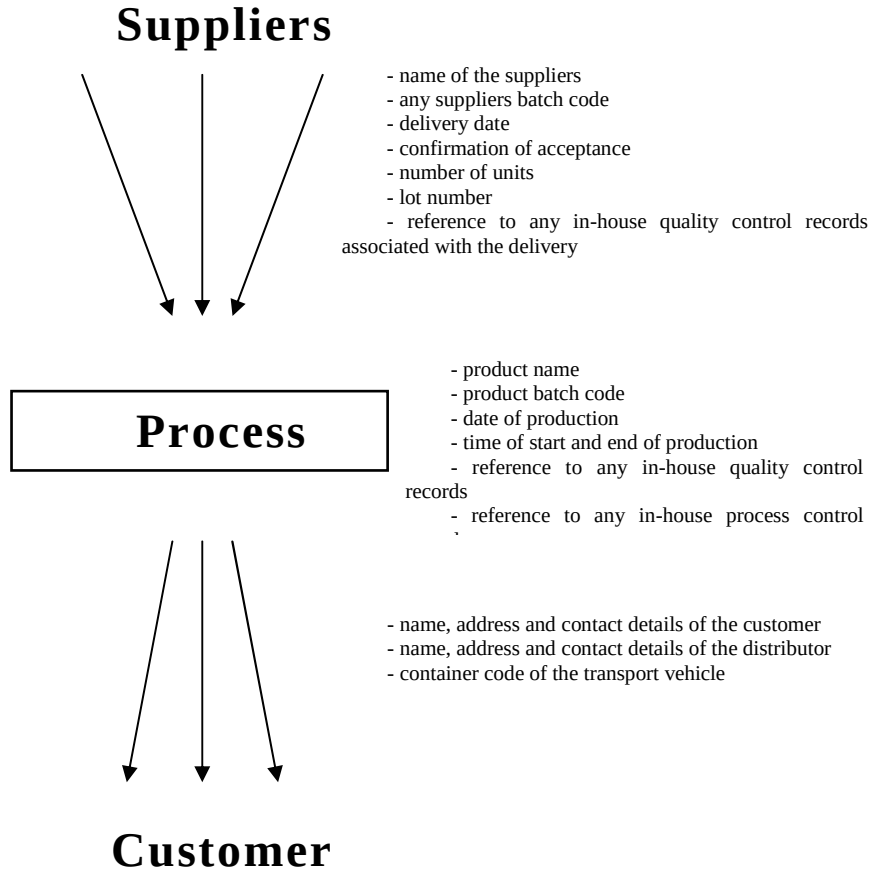

Figure 2. *Components of traceability and information to be recorded (adapted from Dillon and Thompson, 2003).*

The main objective of traceability is to minimize any adverse health effects by a quick and complete recall. For an adequate recall it is necessary that all food products and all of the ingredients used in producing the food are traceable at all stages of production, processing and distribution.

One of the first considerations is to define a lot. Ideally, a lot is a quantity of food produced and handled under uniform conditions. In the commercial sense, a lot is a quantity of product supposedly produced from a single herd of animals and using identical ingredients, slaughtered and processed under the same conditions.

Traceability needs to be available for the following components (Fig. 2):

- supplier traceability: traceability of suppliers and their products entering the business in question;
- process traceability: traceability of foodstuffs through the supply chain;
- customer traceability: traceability of foodstuffs to the immediate customer.

The main elements of each component are summarised in Table 2. Attention must be given to the interface between the three areas above to ensure that the traceability system is seamless.

Table 2. Main elements in a traceability system through suppliers, processor and customers (adapted from Dillon and Thompson, 2003).

SUPPLIERS	PROCESSOR	CUSTOMERS
• each incoming unit of ingredients should carry a means of tracing its source of supply and history • goods inwards documents for each delivery should record all information necessary to maintain traceability from the supplier	• a product batch must be identified • to ensure that a product batch is a true batch, it must be separated by a clean break from other products batches that use the same equipment. Where carry-over is likely, food business should conduct studies to define the likely extent of carry-over, which should be documented • a unique batch code identifier should always be applied to it • each and every saleable unit in the product batch should be coded • internal documentation should accompany the product batch • the traceability codes of ingredients and primary packaging used for a product batch should be recorded and associated with the product batch code • processing and quality records should contain all the necessary information relating to the processing conditions, ingredients used, storage conditions, etc., to allow traceability from the finished product	• a list of all immediate customers, details of the products they purchase and full contact details should be held by food business. The list must be updated regularly • any documentation accompanying the product at the point of sale should contain all the information necessary for traceability to be maintained through the distribution chain • a full list of the products being purchased by the customer with details of each product should be held

3. Traceability in rabbit meat production

Large rabbit industry integration is becoming more important and the development of rabbit meat production is forcing processing plants to improve slaughter capacities by using high-speed and more automated slaughter lines. From the point of view of food safety, as observed in poultry, these changes can lead to higher microbial risks due to possible cross-contamination during preslaughter (crating, transportation, and holding conditions) and processing (skinning and evisceration) operations. The microbial risks are also increased by the higher degree of manipulation needed to produce added-value products as for example the use of meat after grinding and the mix with ingredients of different origin (Mulder, 1999).

Before January 1st 2005, major companies improved voluntary traceability systems and indicators on labels aimed at restoring consumer confidence in meat products (Bernues *et al.*, 2003; Grunert *et al.*, 2004). Few rabbit production companies are highly integrated as each company or organisation virtually controls all the aspects of the rabbit production chain, i.e. (in reverse order) marketing, processing, feed compounding, commercial rearing and breeder management (Fig. 3). The identity of the rabbit carcasses and meat products from a farm can be maintained by collecting the documentation and labelling. The introduction of traceability procedures is more difficult for company or organisation partially or not vertically integrated, because it requires a suitable level of organization within the companies involved in the rabbit chain. A single farm, slaughtering plant or cutting unit would, in fact, have great difficulty in adopting a traceability system independently, or even simply in participating in a system without technical and organisational support.

What follows is a case of the rabbit pathway traceability of a company, or organisation, which is vertically integrated and controls all aspects of rabbit production. However, this approach can be also adopted by each component of the rabbit production chain.

Tables 3 and 4 summarize the procedures and the documents needed in each production step for ensuring traceability of rabbit meat products. It is recommended that documentation be kept for at least two years.

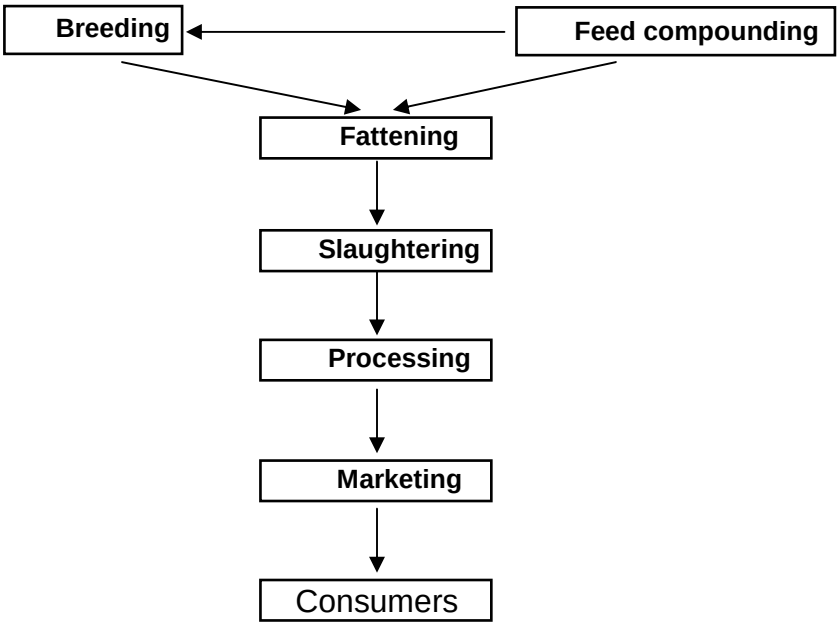

Figure 3. *Food chain of rabbit products.*

3.1. Breeding

Nowadays rabbits reared for commercial purposes belong mainly to breeds or hybrids developed by breeding companies. The economics of animal production prevent the possibilities of cross breeding and back-breeding from grand-parents to commercial levels and indirectly serve to protect the integrity of the traceability. Full records are available at each genetic level. While this extraordinary depth of traceability has been developed for productivity reasons, it can also be used to provide quality and safety guarantees to consumers.

From the perspective of traceability, all rabbits born the same day, in the same house from the same genetic group of rabbit breeders can be considered a single production unit. This means that the synchronisation of births is very important in the rearing management of rabbit does. Traceability procedures are different depending on whether breeding and fattening are conducted in the same (closed-cycle) or separate (open-cycle) farms. Weaned rabbits are usually placed in fattening cages in rabbitries that may contain thousands of rabbits. Considering a closed-cycle farm, the weaned rabbits are directly transferred from the breeding to the fattening house. The adoption of an "all-in all-out" practice could facilitate traceability procedures. In open-cycle farms, weaned rabbits are usually transported by trucks from the breeding to the fattening farm. One possibility could be to send each production unit to one single fattening farm. An accompanying document has to contain the identification of the farm of origin and the map of the location in the trucks of the weaned rabbits.

3.2. Fattening and feed compounding

From a traceability point of view, the main points to check during the fattening period are feeding, medication and vaccination treatments and the chemicals to which rabbits may have been exposed. The identification of feed is given during manufacturing in the feed mill by registering the type and the source of each raw material. After January 1st 2005, companies must also be able to trace feed and feed materials used in feed compounding. An accompanying document for feed should also contain information concerning the storage silos at the feed mill and at the farm, respectively. Care should be taken to avoid possible cross-contamination between unmedicated and medicated feed during compounding, transportation and storage phases.

All animal tests and medications during the animal lifecycle must be administered under veterinary surveillance and recorded. Concurrent storage of paper and electronic data may be used as an alternative method for the collection and storage of data. Technical personnel should check the compliance of the rules concerning feeding, environment and sanitary treatment at least every 2 weeks as well as the documentation records.

3.3. Slaughtering

For good management of slaughter and traceability procedures, the collection of the production units (rabbit flocks) from the farms should be planned well in advance. At the farm, the fattening rabbits must be crated in order to maintain the identification of the production units. The accompanying document must contain the position of crates on the truck if different production units are present. Upon arrival in the holding area of the processing plant, the blocks of crates must be unloaded from the trucks, placed separately and identified by production unit. Each production unit must be separately slaughtered and it is useful to space two consecutive production units by leaving a gap of some minutes as well as by hanging a mark on the processing line with the traceability code.

Table 3. Procedures and documents needed in feed compounding, breeding and fattening for ensuring traceability of rabbit meat products.

PRODUCTION PHASE	Artikel I. PROCEDURES	DOCUMENTS
FEED COMPOUNDING	*Traceability of main raw material components*	Accompanying document of raw materials Document of receipt and storage of raw materials Feed formula and Report of feed compounding Document of traceability of raw materials
	Traceability of additives	Accompanying document of additives (medicaments, integrators) Document of receipt and storage of additives (medicaments, integrators) Feed formula and Report of feed compounding Document of traceability of additives (medicaments, integrators)
	Identification of feed storage silos	Accompanying document of feed
	Identification of production unit of feed	Chemical composition of feed Accompanying document of feed Feed formula and Report of feed compounding Document of receipt and storage of raw materials and additives
BREEDING	*Identification of production unit of weanlings*	Accompanying document of weanlings Document for traceability of does Technical document of farm management
	Identification of feed storage silos	Accompanying document of feed
	Identification of weanling exposed to pharmacological treatments	Recording of medications administered Accompanying document of medications, detergent and sanitizing products Technical document of farm management
	Identification of rabbitry	Accompanying document of delivered weanlings
	Identification of detergent and sanitizing products	Accompanying document of medications, detergent and sanitizing products
FATTENING	*Identification of production unit of fattening rabbits*	Accompanying document of bought weanlings Document for traceability of does Technical document of farm management
	Identification of feed storage silos	Accompanying document of feed
	Identification of rabbits exposed to pharmacological treatments	Recording of medications administered Accompanying document of medications, detergent and sanitizing products Technical document of farm management
	Identification of rabbitry	Accompanying document of delivered fattening rabbits
	Identification of detergent and sanitizing products	Accompanying document of medications, detergent and sanitizing products
	Identification of transport company and vehicle	Accompanying document of bought rabbits with map of disposition

Table 4. Procedures and documents needed in slaughtering, cutting and further processing for ensuring traceability of rabbit meat products.

Production phase	Artikel II. Procedures	Documents
SLAUGHTERING	*Identification of cages in holding area*	Accompanying document of fattening rabbits, Slaughtering schedule Document for check of production units at arrival Signal and mark for production unit identification
	Identification of production units during slaughtering	Slaughtering schedule Accompanying document of fattening rabbits Slaughtering recording document and Traceability coding
	Identification and labelling of each carcass	Slaughtering schedule, Slaughtering recording document, Traceability coding
	Packaging of production units	Slaughtering schedule, Slaughtering recording document, Traceability coding
	Forming of pallets	Slaughtering schedule, Slaughtering recording document, Traceability coding
	Identification of detergent and sanitizing products	Accompanying document of medications, detergent and sanitizing products
	Identification of clients	Accompanying document of products
CUTTING	*Storage and identification of pallets or production unit*	Accompanying document of pallets
	Cutting of carcasses for each production unit	Processing schedule Signals and marks for production unit identification Traceability document for portioned and packaged products
	Packaging	Processing schedule Traceability document for portioned and packaged products
	Identification of box containing packaged products	Traceability document for portioned and packaged products Labels for production unit identification
	Identification of box containing raw meat for further product preparation	Traceability document for portioned and packaged products and further processed products Signals and marks for production unit identification Processing schedule
	Weight and labelling of packaged products	Traceability document for portioned and packaged products and further processed products
	Identification of detergent and sanitizing products	Accompanying document of medications, detergent and sanitizing products
	Identification of clients	Accompanying document of products
FURTHER PROCESSING	*Identification of box containing raw meat for further product preparation*	Traceability document for further processed products Traceability document for raw meat Signals and marks for production unit identification
	Identification of raw materials for further product preparation	Signals and marks for production unit identification
	Preparation and packaging of products	Traceability document for further processed products
	Identification of box containing packaged products	Traceability document for further processed products Labels for production unit identification
	Weight and labelling of packaged products	Traceability document for further processed products
	Identification of detergent and sanitizing products	Accompanying document of medications, detergent and sanitizing products
	Identification of customers and distribution.	Accompanying document of products

3.4. Further processing

The blocks of carcasses can be moved inside the same processing plant or transported to an external plant. The latter case requires accompanying documents. The carcasses sold as whole must be packaged and the labelling must contain information that will vary from country to country, and also the traceability code and some voluntary information concerning farm of origin, place of slaughtering, some characteristics of feeding (no GMO, vegetable diet, etc.). The traceability code may be formed from production line numbers, shift number, time of production, etc., in any combination. This may additionally be encoded within a bar code (Pettitt, 2001).

Major difficulties arise when carcasses are portioned into parts prior to retail packaging. As for processing, the quantities of meat needed for portioning, deboning and further processing should be planned in advance. Any kind of production must be separately conducted for each unit and the products should be packaged in continuum after portioning or deboning. If this is not possible, it has been suggested that group differentiation should be maintained by physical separation and manual records. The packaging and labelling of cut-up and deboned products must be conducted following the same indications given above for the whole carcasses. Traceability must be guaranteed for ready-to-cook products containing additives and further raw materials such as other kinds of meat or vegetables. In these environments, the implementation of process control practises utilising mechanical separation and written documentation (of type described by the International Organization for Standardization in ISO 9000) offers opportunities to maintain such identify system control (McKean, 2001).

3.5. Marketing and measures of recall

In order to comply with legislation, measures of recall of products must be carried out. Notification has to be sent to all members of the network to verify whether the products on the market have reached their market yet so they can take the necessary measures.

To ensure that a product may be recalled effectively in an emergency, the following information should be recorded: time and date of incident, nature of the incident, product affected, what happened to any contaminant, list of relevant tests and analyses carried out and analysis reports. It is also essential to collect the following product information: product code, number of cases affected, batch number, best before/use by date and pallet number.

Using this information the emergency recall team should contact the appropriate company and ask for them to remove and recall all relevantly coded products back to the factory or source of origin of the problem (Dillon and Thompson, 2003).

3.6. Definition of responsibilities

Because of their complexity, companies need specialised managements to oversee different departments. It would be desirable for experts in agriculture, nutrition, veterinary medicine and food science to be employed. Each part of the enterprise (e.g. the feed mill, the breeding flocks, the reproduction and the rearing farms and the processing establishment) must have an assigned manager who is responsible for optimising the output of his section.

4. Future developments

In the past 10-15 years, computer technology has made traceability of food possible in new and innovative ways. Nowadays there are sophisticated meat traceability software systems that can enable producers to track a meat product all the way from animal birth to the supermarket display case and every step along the way (Gledhill, 2002).

Recently, Schwägele (2005) reviewed the available analytical techniques that can detect certain characteristics of (or element in) foodstuffs derived from animal tissue. Some of these techniques can be used to define information regarding origin or history, while others can only be used to confirm the presence of specific components. With respect to traceability, they must give information on animal species, origin, authenticity, age, composition and production systems (including feed). The development of biological identification technologies (Cunningham and Meghen, 2001) and DNA testing (Portetelle *et al.*, 2000) allows for straightforward traceability of individual animals such as cattle and pork (Sancristobal-Gaudy *et al.*, 2000; Arana et., 2002; Maudet *et al.*, 2002; Eggen and Hocquette, 2003; Vazquez *et al.*, 2005). However systems of individual identification are unlikely for commercial rabbits.

Ear-bands and tattoos have traditionally been used to identify individual rabbits at the elite breeding level, principally for research purposes. As each flock/production unit of rabbits has similar status and the existing systems are well developed, individual rabbit identification does not appear to offer any advantages. However the development of innovative techniques, such as intraperitoneal transponder (Pinna *et al.*, 2004), can allow individual identification mainly for highly added - value products (i.e. organic, PDO or PGI rabbit meat). Some trials have been undertaken in poultry on using various automatic methods for identifying individual carcasses by batch code at the grading

line (Fallon, 2001). Inkjet and laser systems have been tested. The laser systems appear to be the more successful, but both systems suffer from consumer resistance to the appearance of the marked part of the carcass. In either case, this identification is rapidly lost as the carcass is further processed. Computer systems for maintaining traceability of batches of product to the farm of origin are likely to be adopted by the companies not already doing so. The sophistication and automation of the systems already functioning in some places will no doubt continue to be refined. The limiting factor of these systems is the situation in which the volume of a particular product produced from a batch of meat falls below the minimum required for industrial handling. In the case of certain specialised products, raw materials may be sourced from many different flocks. These products may in themselves form an ingredient which is added to other batches of products.

5. Conclusions

The compulsory introduction of traceability systems in the EU after 2005 for all feed and food operators represents an opportunity for the European rabbit production chain to improve food safety procedures and restore consumers' confidence.

This adoption must definitively guarantee the ability to effectively remove a single production unit from sale and consumption if any doubt arises as to the status of produce. The ability to recall a limited quantity of produce, based on product traceability, can prevent food diseases, but also reduce costs and even limit brand damage. Moreover the adoption of traceability systems is a part of food safety for companies, who will thus have an instrument to improve all food quality aspects by finding out exactly where problems originate along the production chain.

However the adoption of traceability systems must be possible on a cost basis for both small and big companies. In fact rabbit production chains are sometimes characterized by a high level of fragmentation, low levels of association and lack of structural development in all elements. This could trigger doubts as to the capacity of the rabbit sector to respond to these challenges. So the adoption of a suitable level of organization within companies involved in the rabbit chain and the development of programmes designed to train and assist small producers with the application of traceability procedures are necessary. Also Governmental Authorities must organize an efficient system of inspection to control the compliance of traceability systems by the companies and provide sanctions when rules are broken.

However measures to trace rabbits and rabbit products must be based on an assessment of the risks and be scientifically justified, according to the circumstances, and be no more restrictive of trade than required and applied consistently, including between the country imposing the measure and other countries. Measures that are based on international standards are deemed to be necessary. Otherwise traceability can ensure that products can be linked to their sources while protecting products of declared origin (both geographical and production systems).

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