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Some perinatal endocrine and morphological aspects of canine species

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To myself,

for how passion can become a work

Index

CHAPTER 1

Foreword

1. Foreword

Recently the interest for the canine neonatology, mainly aimed to the improvement of survival after birth and to the correct management of the newborn puppies, is increasing.

In mammals, the newborn represents the result of the prenatal intrauterine development, along which several factors play a crucial role to ensure the fetal growth, well-being, and protection. In this respect, the placenta partially maintains the best environment for the conceptus and it was recognized that fetal fluids, especially the amniotic fluid, contain many important substances involved in fetal growth, development, and well-being.

Both the late pregnancy and neonatal period are considered the most stressful stages for the fetus and newborn, respectively. In fact, an hypothalamic-pituitary-adrenal (HPA) axis activation of the subject occurs during these phases and the consequent cortisol release leads to the final multi-organ maturation in the fetus and even allows the trigger of parturition, whereas in the newborn it is associated to the gradual adaptation to the extrauterine life.

The process of birth represents the transition from the harmless intrauterine life to the outside world, where numerous factors could become causes of neonatal morbidity and mortality. Newborn puppies (as well as kittens) are much less mature than newborns of many other domestic species; therefore, their management is quite challenging for the veterinary practitioner, because of the small size and immature multi-organ functions. In particular, the immune system is not fully developed, so that, at the beginning of their extrauterine life, all mammals newborns depend on the passive immunity acquired by the mother, through the placenta and/or colostrum.

Along the neonatal period, several physiologic changes normally take place, ensuring the maturation and adaptation of the newborn. Concurrently, also the whole skeleton conformation undergoes a gradual evolution. That said, canine species is characterized by a wide variety of breeds encompassing many body shapes and sizes; additionally, the growth speed appears extremely rapid and influenced by the breed, sex, genetics, environment, endocrine as well as metabolic factors, diet, and pathologic conditions.

Aims of the thesis

Because of the recognized importance of the perinatal challenging period for the survival and health of the offspring, the present thesis was aimed to investigate some aspects of the perinatal phase, i.e. the time ranging between the last third of gestation until the first 30 days after birth, in the dog.

To the author knowledge, the amniotic and allantoic composition throughout pregnancy, as well as the role of the amniotic and allantoic compounds involved in the fetal growth and development, and in preparation for birth, were not yet fully investigated in canine species. Furthermore, the hormonal mechanisms which dominate both the final phase of gestation and the beginning of the extrauterine life were not fully clarified in dogs. The present thesis was aimed, first of all, to provide a deeper knowledge about fetal fluids composition, by evidencing the presence and the concentrations of some factors (IGF-I, NEFA, IgG, and lysozyme) necessary for fetal and neonatal growth, development, and well-being, and to evaluate the HPA axis activation in the newborn, by measuring cortisol levels in many biological matrices. Both these studies were carried on in the respect of the animal welfare, through not-invasive techniques. In fact, IGF-I, NEFA, IgG, and lysozyme levels were assessed in fetal fluids collected during elective cesarean sections, performed because of the high risk of dystocia or previous history of troubles at parturition, whereas cortisol concentrations were analyzed in both hair and nails collected by born dead newborn puppies or puppies dead spontaneously within 7 days of age. The present thesis represents only a starting point; further researches are needed to improve the clinical application, for instance by evaluating the possible correlations between both IGF-I and NEFA and fetal growth diseases, or by assaying cortisol levels in healthy and sick newborn puppies not only at birth, but during the neonatal phase and, if possible, even later, to monitor the last period of intrauterine development as well as the neonatal adaptation after birth.

The neonatal period represents another really interesting phase, along which indispensable physiologic changes have to take place to ensure the survival after birth. In particular, the present thesis was aimed to investigate the real bacterial involvement in canine neonatal mortality, since the bacterial infections are considered as the second main cause of neonatal loss in dogs. Despite the impact

of bacterial infections on neonatal death, only few studies were published about the incidence of septicaemia in newborn puppies.

Additionally, in canine species, also the information availability about the skeletal development are limited, above all within the first month of age. The previous studies about the evaluation of the ossification centers appearance and fusion are difficult to compare each other because, in most cases, the breeds enrolled, age and method of examination, anatomical compartments investigated, as well as the aim of the research, were not homogeneus. Thus, it could be useful the creation of a radiographic data-base concerning the ossification centers appearance during the first month of age in newborn puppies, in order to investigate the normal growth, as well as to detect a new easy tool to estimate their real age, above all in case of illegal import. At this regard, the third aim of the present thesis was to investigate the timing appearance of hindlimb ossification centers, as well as the radiographic and anatomical morphometry of the hindlimb long bones, skull, and body, in large and giant sized puppies dead spontaneously within the first 30 days of age.

CHAPTER 2

Canine pregnancy

2. Canine pregnancy

2.1 Fertilization

The term "fertilization" indicates the union of male and female pronuclei, which marks the beginning of the embryonic development. In most bitches, this process occurs in metaphase II oocytes, from 90 hours after ovulation (Reynaud *et al.*, 2005).

In the oviduct, spermatozoa change their motility pattern from a progressive, linear motility into a hyperactive frenzied motion, probably in order to facilitate the interaction with the oocyte. To date, it is well known that spermatozoa show particular proteins on their plasma membrane surfaces, which bind specifically to the glycoproteins of zona pellucida. Then, the acrosomal reaction, during which the fusion between the spermatozoa plasma membrane and the outer acrosomal membrane occurs, leads to a vesiculation that allows for both the digestion and penetration of the zona pellucida by the acrosomal enzymes. When the spermatozoon reaches the perivitelline space, the oocyte plasma membrane fuses with the equatorial segment of spermatozoon. Thus, the oocyte undergoes several changes aimed to the early embryogenesis, such as the cortical reaction. During the first and second meiotic divisions, cortical granules fuse with the oocyte plasma membrane, releasing their contents into the perivitelline space to prevent the polyspermy. After the inclusion of the spermatozoon nucleus within the oocyte cytoplasm, the fusion between the male and female pronuclei, or syngamy, can occur (Senger, 2003).

2.2 Embryogenesis

A successful preattachment phase during pregnancy requires the progression from zygote to blastocyst, as well as the development of a functional trophoblast. Canine embryonic and fetal development occurs within 61 days from the fertilization, a relatively short interval compared to many other mammals (Pretzer, 2008).

2.2.1 From the zygote to the blastocyst

The "period of the ovum" defines the interval between days 2 and 17 after LH peak (Pretzer, 2008). Shortly thereafter the syngamy, the embryo, now defined

"zygote", undergoes several cleavage divisions. The reaching of the uterotubal junction takes place in 7-10 days for canine zygotes compared to 3-4 days typical of the other species (Reynaud *et al.*, 2005; Pretzer, 2008; Reynaud *et al.*, 2012). The first mitotic division generates a two-celled embryo, formed by blastomeres, each of them representing more or less one-half of the zygote; subsequently, each blastomere is subjected to additional divisions, giving rise to the morula. To the author knowledge, the data available concerning the oocytes maturation timing in the bitch are still controversial (Tsutsui, 1975; Renton *et al.*, 1991; Bysted *et al.*, 2001; Concannon *et al.*, 2001; Reynaud *et al.*, 2005; Reynaud *et al.*, 2012). The continuous divisions of the morula cells lead to a separation between inner and outer cells, with the consequent blastocyst development. The blastocyst is formed by an inner cell mass, a cavity called blastocoele, and a single outer layer of cells, named trophoblast. The inner cell mass of the blastocyst will form the embryonic body, whereas the trophoblastic cells will give rise to the chorion, the fetal component of the placenta. During the morula stage, some fluid can accumulate inside, thanks to an active sodium pump and particular junctions, which allow for intercellular communication and alter the cellular permeability. The combination among the pression increase caused by the fluid accumulation in the blastocoele, the degradation of the zona pellucida by some proteolytic enzymes, released by the trophoblastic cells, and the blastocyst contractions, causes the rupture of the zona pellucida, with following hatching of the blastocyst (Senger, 2003). The timing of the preattachment embryogenesis depends on species; in the bitch, the blastocyst hatching from the zona pellucida usually occurs 13-15 days after ovulation (Senger, 2003), even if this process can be delayed until day 19 after LH surge (Concannon *et al.*, 2001).

The passage of the developing embryos into the uterus can occur as early as 16-cells stage, but more commonly as morulae or early blastocysts (Pretzer, 2008; Reynaud *et al.*, 2012), 8-10 days after ovulation (Reynaud *et al.*, 2012). An intrauterine and transcornual migration of the blastocysts is possible from day 12 to 17 after LH peak (Pretzer, 2008). The beginning of the fixation and implantation was suggested by day 17-19 post LH surge (Pretzer, 2008) or 16-17 after ovulation (Reynaud *et al.*, 2012).

2.2.2 From the implantation to the organogenesis

The "period of the embryo" includes the interval ranging from 19 to 35 days after LH surge. The most important event within this phase is defined "gastrulation", along which the blastula becomes a trilaminar structure consisting of an outer ectodermal, a middle mesodermal, and an inner endodermal layer (Pretzer, 2008). During the early embryonic development, the endoderm forms under the inner cell mass and grows downward on the inner surface of the trophoblast, giving rise to the yolk sac, a transient extraembryonic membrane that regresses as the conceptus growth progresses. Simultaneously, the mesoderm develops between the endoderm and the embryo, surrounding the yolk sac and pushing against the trophectoderm (previous trophoblastic cells). Additionally, the mesoderm begins to fold upward, forming the amniotic folds around the embryo. While the amniotic folds fuse above the embryo, the mesoderm fuses with the trophectoderm cells forming the chorion, giving rise to a double sac around the conceptus. The inner sac is defined amnion and it consists of trophectoderm and mesoderm; it creates the amniotic cavity, filled with fluid responsible for the mechanical protection of fetus. The chorion completely surrounds the conceptus. At the same time, the allantois, a diverticulum from the primitive gut which collects embryonic wastes, develops, surrounded by the mesoderm. As the embryo grows, the allantois continues to expand and fuses with the chorion, forming the allantochorion. The allantochorionic membrane represents the fetal contribution to the placenta and provides the surface for the attachment to the endometrium (Senger, 2003).

The ectodermal, mesodermal, and endodermal layers are indispensable for both the extraembryonic membranes formation and the following embryonic organogenesis. Especially, ectoderm forms the skin epidermis and the neural tissue; endoderm differentiates into the inner layer of the gastrointestinal and respiratory tracts, whereas the urogenital, circulatory, as well as muscular skeletal systems, derive from the middle mesoderm (Pretzer, 2008). Regarding the reproductive organs, the ectodermal layer gives rise to the mammary glands, hypothalamus, pituitary lobes, caudal vagina, vestibule, and penis or clitoris, whereas the mesodermal layer differentiates into the gonads, uterus, cervix, cranial vagina, epididymis, and accessory sex glands (Pretzer, 2008).

The vesicles can be observed ultrasonographically as 1-2 mm round anechoic structures as early as day 19 after LH peak, whereas the canine embryo by days 22-23 and the heartbeat on days 23-24. The development of the dog embryo starts with the head folds and neural tube closure, and continues with the somite formation, appearance of branchial clefts, lens and otic placodes, cardiac bulge, and finally growth of limb buds. An early embryo is characterized by a minimal anatomic differentiation: only the heartbeat flickering motion and the anechoic area in the head can be detected by ultrasounds. The first detectable abdominal viscera are the stomach and urinary bladder, on days 29-33 and 31-35 respectively. The skeleton appears as hyperechoic structure on days 29-33 (Pretzer, 2008).

At 23 days of gestation, canine embryo is 10 mm in length and a prominent thoracic limb bud, otic and lens placodes, and both mandibular and maxillary processes are evident. By 25 days, the embryo is 14 mm long; at this stage, the mammary ridge is present, whereas the vertebral structures and the dental lamina are forming. In 28 days embryo (17 mm), the process of ossification is occurring in the mandible, maxilla, frontal bone, and clavicle. By 30 days, the embryo appears 19 mm in length; the eyelids and external ear are developing, as well as sensory hair on the muzzle, chin, and eyebrows. Simultaneously, the intestine fills the available abdominal cavity and appears herniated through the umbilical stalk. Also nipples, forelimb digits, and a prominent genital tubercle are detectable. The 33 days embryo (27 mm) is characterized by the ossification of nasal, incisive, palatine, zygomatic, parietal bones, ribs, and the midshaft of several long bones limbs. Additionally, canine teeth are developing, the fusion of palatal shelves is visible, and the hindpaws digits appear distinct (Pretzer, 2008).

2.2.3 Maternal recognition of pregnancy

Luteolysis must be prevented and progesterone maintained sufficiently high to carry on the established pregnancy. Normally, the maternal recognition of gestation, through which the fetus signals to the mother its presence, represents an important step. In the bitch, this is not necessary since, contrary to the females of the other species, the lifespan of corpora lutea is the same during and without pregnancy (Senger, 2003).
However, pre-implantation canine embryos are able to express enzymes and cytokines that regulate the trophoblast growth and promote some endometrial

changes (Schäfer-Somi, 2012). In particular, Schäfer-Somi *et al.* (2008) suggested an active role of the embryo before and during the invasion, reporting some genes expression in canine pre-implantation uterus and embryo. In the bitch, it was demonstrated that pre-implantation uterus and dioestrus uterus express similar factors; however, the factors exclusively detected in pregnant uterus are interleukin-4, CD8, and interferon γ, which modulate the intrauterine response towards a predominance of Th2 cells, aimed to the maintenance of pregnancy in other species. At placentation sites, also the expression of insulin-like growth factor-II (IGF-II) and granulocyte-macrophage colony-stimulating factor (GM-CSF) was reported (Beceriklisoy *et al.*, 2009).
Both the intrauterine growth and angiogenesis were already investigated (Beceriklisoy *et al.*, 2009; Schäfer-Somi *et al.*, 2009; Schäfer-Somi *et al.*, 2012), evidencing an important increase of some growth and angiogenesis factors during pre-implantation and implantation (Schäfer-Somi *et al.*, 2012). Additionally, the progesterone receptors were observed to be down-regulated inside the pregnant uterus except at placentation sites to carry on the gestation; conversely, a significant up-regulation of the intrauterine leukemia inhibitory factor (LIF) was documented (Schäfer-Somi *et al.*, 2009).
The influence of maternal hormones on the embryo growth and secretion of cytokines, growth factors, and enzymes still has to be investigated (Schäfer-Somi, 2012).

2.3 Placentation and fetal fluids

2.3.1 Placenta

One of the most important events during embryonic development is the formation of the extraembryonic membranes, which are essential for the metabolic, gaseous, and hormonal exchange, and hence, for the embryo survival.

The term "implantation" means the attachment of the placental membranes to the endometrium. The placenta is a transient organ, necessary for both metabolic interchange between the conceptus and dam, and endocrine control (King, 1982; Chucri *et al.*, 2010). Furthermore, it holds a great immunological role in the antibodies transfer, tolerance and regulation of fetal development, cytokines release, and in the helper and cytotoxic lymphocytes (Michelon *et al.*, 2006; Chucri *et al.*, 2010).

The fetal contribution to the placenta is the chorion, characterized by small projections, the chorionic villi, on its surface. According to the chorionic villi distribution, placentae are classified in diffuse, zonary, discoid, and cotiledonar. The zonary placenta is typical of dogs and cats, and it is formed by a transfer zone, a pigmented zone, and a relatively nonvascular zone. In this type of placenta, a band of tissue surrounds the middle of the conceptus, where the transfer of nutrients occurs (Miglino *et al.*, 2006). The pigmented zone consists in an highly pigmented ring at either end of the central zone, which represents local regions of marginal hematomes (Miglino *et al.*, 2006), probably involved in the iron transfer to the fetus in both dog and cat (Leiser and Enders, 1980; Leiser and Kaufmann, 1994). The third region is represented by the transparent zone on the distal ends of the chorion, likely responsible for the direct absorption of substances from the uterine lumen (Miglino *et al.*, 2006).

Miglino *et al.* (2006) deeply investigated the features of the fetal membranes and the degree of elaboration of both amnion and allantois in canine placentae from 20, 24, 35, 45, and 55 days of pregnancy. At day 20 of gestation, the yolk sac was prominent compared to the embryo and completely vascularized. An avascular amnion entirely surrounded the embryo. The internal surface of the early placental girdle did not show blood vessels. At day 24, the embryo became more distinct and the fetal membranes easy to distinguish. The amnion contained translucent amniotic fluid, whereas the allantoic sac was full of a clear yellow fluid. The central part of the allantoic sac represented the placental contacts of the endometrium; it surrounded the embryo almost totally in canine species. Two hematomas started to appear from 22 to 25 days of pregnancy, as green borders of the placental girdle, originated by blood extravasation from maternal capillaries. Between 25 and 30 days of gestation, the blood vessels from the umbilical cord began to supply both the yolk sac and placental girdle. The amnion was supplied by few very fine vessels of allantoic origin, which allow for the nutrient diffusion. Fetal and maternal tissues of the placental girdle began to indent each other, especially from day 35 onwards, and involved particularly the fetal and maternal vascular systems. Up to day 45 of pregnancy, the allantoic sac appeared prominent because of its increased size and developing vasculature. At day 53, the fetus was almost entirely developed inside the amnion, which was completely enveloped by the allantois (Miglino *et al.*, 2006).

According to the number of tissue layers between maternal and fetal blood, placentae can be classified as epitheliochorial, syndesmochorial, endotheliochorial, and hemochorial. The endotheliochorial placentation is typical of dogs and cats, and it is characterized by a complete erosion of both the endometrial epithelium and underlying interstitium, with following exposure of maternal capillaries to the epithelial cells of the chorion (Senger, 2003; Aralla *et al.*, 2013).

2.3.2 Amniotic fluid

Production and removal

Amniotic fluid (AF) is a wonderfully complex and unique fluid that nourishes and protects the fetus. During embryogenesis, its volume increases faster than embryo and later it provides mechanical protection and essential nutrients and molecules for the growing fetus (Underwood *et al.*, 2005). In early fetal phase, the biochemical composition of the AF in humans is really similar to the fetal extracellular fluid, probably due to transudation across the fetal skin before keratinization (Liu *et al.*, 2008). In human beings, a fast bi-directional diffusion occurs between the fetus and AF across the not keratinized skin and the surfaces of the amnion, placenta, and umbilical cord, absolutely permeable to water and solutes (Underwood *et al.*, 2005). The same phenomenon was reported in sheep, that showed an important transfer of water and electrolytes across the skin until late development. On the contrary, little is known in other domestic species (Fresno *et al.*, 2012).

After the fetal skin keratinization, the human AF volume is determined by several factors, which include the AF circulation. Amniotic fluid originates from secretions by the respiratory tract, oral cavity, gastrointestinal tract, as well as by excretion of fetal urine (Underwood *et al.*, 2005). Its removal predominately occurs by fetal swallowing, even if a significant intramembranous pathway promotes the passage of fluid and solutes from the amniotic cavity to the fetal circulation. Nevertheless, the AF volume is only minimally affected, probably due to other control mechanisms, such as the compensation, hormonal changes, and uterine perfusion (Underwood *et al.*, 2005). This was documented by the fact that fetal esophageal or intestinal atresia causes polyhydramnios in only 50% and 60% of cases, respectively. Compensation is evident in sheep where esophagus ligation leads to an increased absorption of AF into the fetal circulation without

any variation in the total fluid volume. Several researches suggested that passive diffusion is only partially responsible for the intramembranous fluid absorption and that many solutes diffuse in the opposite direction, from fetus to AF. Brace *et al.* (2004) reported that probably much larger shifts of fluid and solutes occurred by the AF transfer into the fetal circulation perhaps via a trans-cellular vesicular transport mechanism. Vascular endothelial growth factor (VEGF) seemed to mediate this process in the ovine fetal membranes (Cheung, 2004). Recent studies performed on fetal sheep during late pregnancy elucidated the regulation mechanisms of the AF volume; interestingly, it is mainly regulated by modulating the rate of intramembranous absorption of the amniotic water and solutes into the fetal vessels, although also fetal urine production, lung fluid secretion, and fetal swallowing are considered essential amniotic inflows and outflows (Brace *et al.*, 2014; Brace and Cheung, 2014).

It is likely that even the hormonal changes play a crucial role in AF volume regulation. In early gestation, there is not a significant number of receptors for estrogen or progesterone in fetal membranes. However, as pregnancy progresses, receptors for decidual prolactin are widely expressed by both fetal and maternal tissues. It was ascertained that, in humans, decidual prolactin has an effect on amniotic permeability, despite this is probably not the only hormonal or growth factor-dependent mechanism (Underwood *et al.*, 2005).

Finally, uterine perfusion affects AF volume. Maternal dehydration induces an increased fetal plasma osmolality, with following increased fetal production of arginine vasopressin. The result is represented by an increase in the osmolality of both fetal urine and AF. The direct injection of arginine vasopressin into ovine AF causes an increase in fetal urine and AF osmolality, besides a decrease in fetal urine output; nevertheless, AF volume does not change, suggesting a reverse intramembranous flow from the isotonic fetal circulation to the hypertonic AF (Mann *et al.*, 1996).

Nutritive properties

Amniotic fluid contains several nutrients essential for fetal development and well-being, such as carbohydrates, proteins, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones. Before the fetal skin keratinization, amino acids diffuse from the placenta and fetal circulation into AF. Later in gestation, diffusion through the placental membranes continues and is increased by fetal urinary

excretion of amino acids. Some amino acids are more present in AF than in maternal serum, such as taurine in humans, whereas most show lower levels in AF compared to maternal and fetal blood (Underwood *et al.*, 2005). Glutamine appears particularly important in rapidly dividing cells, as demonstrated by its uptake from the AF by the intestine in fetal sheep (Bloomfield *et al.*, 2002). Arginine also keeps a crucial role in fetal and placental development; indeed its metabolites work as key regulators of placental angiogenesis, trophoblast growth, and embryogenesis. In sheep, the levels of arginine and its metabolites increase rapidly in both amniotic and allantoic fluids in early pregnancy and remain elevated in AF throughout gestation. As gestation progresses, the swallowed metabolites in AF promote the proliferation and differentiation of intestinal epithelial cells (Kwon *et al.*, 2003).

The role of swallowed carbohydrates and lipids in AF remains still controversial (Underwood *et al.,* 2005). In fact, infusions of dextrose or dextrose with amino acids directly into AF in case of growth-restricted rabbit fetuses did not improve fetal growth, whereas an infusion of bovine AF supported organ and somatic growth (Buchmiller *et al.*, 1994). Furthermore, in fetal rabbits with esophagus ligation, the amniotic infusion of glucose or glucose with amino acids enhanced organ weights and fetal growth (Mulvihill *et al.*, 1985). In the attempt to demonstrate the nutritive value of fetal swallowing, the esophageal ligation in fetal rabbits was performed to prevent swallowing, followed by some infusions into the gut distal to the ligature. Those animals infused with lactated Ringer solution showed poor gut development, whereas those infused with bovine AF had more normal gut maturation (Mulvihill *et al.*, 1986). Also in fetal sheep, improved fetal organ growth was obtained by esophageal infusion of AF (Trahair *et al.*, 2000). Additionally, trophic effects of AF were proved on cultured human fetal small intestinal cells. These studies suggested that growth factors found in AF keep a primary role in fetal growth and development: among these, the epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), transforming growth factor beta-1 (TGF-β1), insulin-like growth factor I (IGF-I), free fatty acids (FFA), erythropoietin (EPO), and granulocyte colony-stimulating factor (G-CSF) (Hagenfeldt and Hagenfeldt, 1976; Urban and Iwaszkiewicz-Pawlowska, 1986; Underwood *et al.*, 2005).

Protective function

Amniotic fluid plays a double protective role, by providing a supportive cushion and keeping a significant immune defensive role. Many of the substances belonging to the innate immune system were detected in AF and they are thought to have significant antimicrobial properties; among these, the α-defensins, lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, psoriasin, and cathelicidin are included. These potent antimicrobials exerted broad-spectrum activity against several infectious agents. The α-defensins seem to be the most important; in fact, they are present in significant concentrations in AF of women without evidence of infection. Their AF levels increase in case of preterm labor, preterm premature rupture of membranes, and chorioamnionitis. Lactoferrin was detected in human milk, as well as in human AF at 20 weeks gestation. Its high concentrations were found in case of preterm labor and amnionitis. Lactoferrin has both bacteriostatic and bacteriocidal activity, since its enzymatic digestion at acid pH releases a potent microbicidal peptide called lactoferricin, that showed antimicrobial effects against viruses, protozoa, and fungi (Underwood *et al.*, 2005).
In mammals, the passive immunity from maternal antibodies represents a vital component of the immune protection to prevent neonatal diseases, as the neonatal immune system is not fully efficient at birth. Compared to humans, in which a significant amount of immunoglobulins are transferred transplacentally, dogs have an endotheliochorial placenta with four layers separating fetal and maternal blood. This type of placentation results in very little maternal immunoglobulin transfer to the fetus, with reported transplacental immunoglobulin passage ranging from 5% to 10% (Tizard, 2009; Chucri *et al.*, 2010; Evermann and Wills, 2011).

The protective activity of the "cellular" innate immune system within AF was less well clarified. The presence of mononuclear phagocytes in AF are limited in normal gestations, whereas their number increases in cases of neural tube defects. Normally, the neutrophils are not identified in the AF of healthy fetuses, but represent a useful marker of fluid infection. Granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) were assessed in AF of healthy term and preterm fetuses. Increased concentrations of

G-CSF were noted in the serum of women with subclinical chorioamnionitis, in the cord blood of neonates with infection, fetal distress, premature rupture of membranes, and meconium staining of AF, as well as in the AF, neonatal urine, and bronchoalveolar fluid of neonates with intra-amniotic infection (IAI). It is unknown if G-CSF and M-CSF play a preventive defense role in the AF or are just excreted during infection (Underwood *et al.*, 2005).

Diagnostic role

Amniocentesis was regarded a valuable tool in assessing fetal well-being since the 1970s. It is commonly offered to women who will be at least 35 years old at the time of full-term delivery or who have other risk factors for a chromosomal abnormality. Assessment of AF keeps an important role also in the prenatal diagnosis of neural tube defects and inborn errors of metabolism, as well as hematologic and genetic diseases. Furthermore, AF investigations were performed on patients with preterm labor and/or premature rupture of membranes to investigate possible IAI. At this regard, AF indicators strongly suggestive of infection include high concentrations of matrix metalloproteinase (e.g., MMP-9), interleukins (e.g., IL-6 and IL-1b), tumor necrosis factor (TNF-α), G-CSF, elevated white blood cell count, low glucose, and the presence of bacteria. In human gestation, amniocentesis represents an helpful tool also in prenatal diagnosis of cytomegalovirus, toxoplasma, and parvovirus B-19 infection. Assessment of fetal lung maturity, through the determination of the lecithin/sphingomyelin ratio and/or the presence of phosphatidyl glycerol in AF became a well-accepted procedure. Nevertheless, other superior techniques were more recently proposed for evaluation of fetal lung maturity, such as the detection of lamellar body counts in AF, the surfactant to albumin ratio in AF, and electrical conductivity of AF (Underwood *et al.*, 2005).

2.3.3 Allantoic fluid

The principle mechanisms for the accumulation of allantoic fluid (AL) during the early human pregnancy include probably the transmembrane transport and the secretory activity of the extra-embryonic membranes. However, in late gestation AL originates mainly from the mesonephros, metanephros, and kidney secretions, becoming more similar to fetal urine. Towards the end of pregnancy,

fetal urine is diverted into the amniotic fluid from the allantoic sac through the urethra, since the urachus occludes progressively (Li *et al.*, 2005).

2.3.4 Fetal fluids in Veterinary Medicine

The biochemical composition of both AM and AL was widely investigated in the past in Veterinary Medicine, focusing in particular on bovine (Baetz *et al.*, 1976; Wintour *et al.*, 1986) and ovine (Wales and Murdoch, 1973; Georgiev, 1975; Wintour *et al.*, 1986). Several researches evidenced that the biochemical and metabolic processes taking place in the fetus lead to systematic changes in fetal fluids volume and composition, above all in amniotic fluid, as gestation progresses, reflecting putative variations in metabolic and transport activity (Baetz *et al.*, 1976; Wintour *et al.*, 1986; Li *et al.*, 2005; Peter, 2013).

Interestingly, the biochemical constituents of bovine fetal fluids were reported to change between day 115 and 265 of gestation (Baetz *et al.*, 1976). On the contrary, in sheep it was documented that the fetal fluids composition varies also in early pregnancy, between day 22 and 44 (Wales and Murdoch, 1973). Nevertheless, fetal fluids homeostasis was still not fully clarified in cattle. The fetus maintains its plasma volume by balancing the volume and composition of both AM and AL, through continuous exchange between maternal and fetal circulation. It is well known that several hormones are likely involved in the regulation of fetal fluids homeostasis, but these hormonal regulators work only when the fetus becomes able to synthesize them. Before this period, the electrolytic composition of fetal fluids must be regulated by maternal hormones or autocrine/paracrine factors (Li *et al.*, 2005). In cattle, during the first trimester of pregnancy, AL accumulation appears faster than AM formation, but after this phase the amniotic volume exceeds the allantoic one until approximately 150 days of gestation. Later, the AL volume increases rapidly, whereas AM accumulates more slowly (Arthur, 1957; Wintour *et al.*, 1986; Peter, 2013). Transmembrane transport and secretory activity of the extraembryonic membranes probably represent the major mechanisms responsible for fluids accumulation before the placentomes formation. With further development, the secretions from the mesonephros, metanephros, and kidneys contribute to the AL composition, whereas secretions from the buccal cavity, respiratory tract, gut, and not yet keratinized fetal skin mainly give rise to the AM (Baetz *et al.*, 1976; Wintour *et al.*, 1986). The most recent researches in bovine species were

aimed to investigate the biochemical composition and amino acids profile of fetal fluids in case of somatic cell nuclear transfer pregnancies (Li *et al.*, 2005; Zhou *et al.*, 2014).

In the last years, several studies were performed about fetal fluids characteristics in mares. Among these, Zanella *et al.* (2014) determined the biochemical profile of both AM and AL in mares during initial, mid, and final phases of gestation, whereas Pirrone *et al.* (2012) investigated the amniotic fluid and blood lactate concentrations in mares and foals along the early post-partum period, to verify the usefulness of this parameter in the evaluation of the foal health. In fact nowadays, the researches on fetal fluids are directing toward the detection of some substances that could have a diagnostic role in some gestational or neonatal pathologies (Pirrone *et al.*, 2012; Canisso *et al.*, 2015).

Concerning the small carnivores, to the author knowledge, only one recent study provided some essential data about the feline fetal fluids (Fresno *et al.*, 2012), since the biochemical and electrolyte composition, as well as their role in fetal metabolism, were not previously documented neither in dog nor in cat. Based on this research, feline fetal fluids composition does not represent the result of simple filtration from maternal blood, since the fetus seems to be actively involved in the final biochemical characteristics of both AM and AL throughout pregnancy. Amniotic and allantoic fluids tend to have a similar biochemical composition in cat, probably due to the poor vascularization of amnion and to the diffusion from allantois vessels to amniotic cells. Also in feline species, some variations in fetal fluids composition occur along gestation, as the reflex of changes in metabolic and transfer activity and differences in the contribution of both fetal and placental tissues to the amniotic and allantoic compartments.

Thus, the fetal fluids remain an interesting topic to better document in canine species.

2.3.5 Endocrine functions of placenta

In mammals, placenta is the major endocrine organ during gestation, as it produces several hormones aimed to stimulate ovarian function, maintain pregnancy, influence fetal growth, stimulate mammary function, and assist in parturition. First of all, progesterone secretion is mandatory to stimulate the endometrial glands secretion, as well as to inhibit the myometrial contractions.

Blood progesterone levels increase gradually in pregnant female, but the timing of progesterone peak and its absolute concentrations vary significantly among species. During early pregnancy, progesterone is always produced by the corpus luteum, even if the following maintenance of gestation depends on species. In pregnant bitch, the corpus luteum is the only source of progesterone (Verstegen-Onclin and Verstegen, 2008; Papa and Hoffmann, 2011; Kowalewski, 2012) and its function is regulated by several species-specific mechanisms, among which the independence of gonadotropic support in the first third of dioestrus (Kowalewski, 2012). Recently, it was documented that PGE2 represents one of the most important luteotropic factors, but afterwards prolactin becomes the main one (Kowalewski, 2012). Concerning the prolactin, Kowalewski *et al.* (2011) strongly suggested that this hormone could play a crucial role not only in maintaining the canine corpus luteum, but also in regulating the placenta function.

In humans, the fetal membranes represent one of the major sites of both prostaglandins synthesis and metabolism (Myatt and Sun, 2010). Prostaglandins have an important role in the initiation and maintenance of labor (Gibb, 1998; Myatt and Sun, 2010), since they are powerful stimulants for the pregnant myometrium. The amount reaching the myometrium depends on the expression of both the prostaglandin synthases (PGHS) in amnion and chorion and 15 hydroxy prostaglandin dehydrogenase (PGDH) in chorion trophoblast, which balance the synthesis and metabolism, respectively (Myatt and Sun, 2010). At this regard, several authors showed that very little amount of prostaglandin can pass the fetal membranes without being converted to an inactive metabolite (Nakla *et al.*, 1986; Bennett *et al.*, 1990; Mitchell *et al.*, 1993). Conversely, at term labor, the increased prostaglandin synthesis, as well as the low PGDH activity and expression in chorionic trophoblast, were demonstrated (Pomini *et al.*, 2000). The influence of corticotropin-releasing hormone (CRH) (McKeown and Challis, 2003), cortisol, and progesterone (Patel *et al.*, 2003) on the regulation of PGHS and PGDH activity was widely studied in both norüal and pathologic pregnancies (Van Meir *et al.*, 1996; Casciani *et al.*, 2008).

Among the several placental products, also estrogens and relaxin are included, above all towards the end of gestation. Relaxin can be secreted by placenta and/or ovary depending on species. In the bitch, it reaches detectable plasma

concentrations at approximately 25-30 days of pregnancy (Concannon *et al.*, 2001) and peaks between days 40 and 50 (Linde Forsberg, 2010).

The placenta serves also as a metabolic exchange organ between the fetus and dam. Gases and water pass by simple diffusion, whereas active transport pumps were detected in placenta for sodium, potassium, and calcium. Glucose and amino acids are transported by facilitated diffusion. Maternal proteins do not cross the placental barrier, except some immunoglobulins, as well as the lipids. Anyway, the fetus is able to synthesizes the most proteins from the amino acids transferred by the dam. Additionally, the placenta hydrolyzes triglycerides and maternal phospholipids to synthesize new lipids for the conceptus. Large peptide hormones do not cross the placenta, contrary to other ones with a smaller molecular weight, such as steroids, thyroid hormone, and catecholamines, that can do it easily. Vitamins and minerals can be transferred to the fetus at variable rates (Senger, 2003).

2.4 Fetal growth

2.4.1 The period of fetus

The "period of fetus" refers to the interval from day 35 of gestation until birth. A 35 days fetus can be finally recognized as canine because of some characteristic external features, such as the pigmentation development, growth of hair and claws, eyelids closure and fusion, growth of external ear, trunk elongation, and sexual differentiation (Pretzer, 2008). By day 40, the eyes closure and lids fusion were observed; also the elimination of the physiological umbilical hernia was detected, as well as the claws formation on all digits. At 45 days of gestation, the color markings appear and the body hair begins to grow. By 55 days, all deciduous teeth are calcified. The last bones to ossify at 57 days include the basihyoid, sacral wings of S1, and talus (Pretzer, 2008).

2.4.2 Endocrine regulation

Several hormones are able to affect the fetal growth, acting on both tissue accretion and differentiation, above all along late gestation. Their actions may partially be mediated by other growth factors (insulin-like growth factors, IGFs) (Fowden, 1995). IGFs exert profound effects on somatic growth and cellular proliferation of many tissues, including the placenta (Fowden, 2003). Both IGF-I

and IGF-II are expressed in fetal tissues and present in fetal circulation, with higher levels of IGF-II during late pregnancy. The expression of IGFs genes is specifically regulated in each tissue and can be affected by nutritional and endocrine conditions in utero. Deletion of these genes retards the fetal growth (Anthony *et al.*, 1995); conversely, an overexpression leads to the fetal overgrowth (Fowden, 2003). Furthermore, IGFs affect the growth of individual fetal tissues and influence the utilization of nutrients by fetal and placental units. IGFs circulating concentrations and tissue expression are reduced by undernutrition and deficiency of nutritionally sensitive hormones, such as insulin, thyroxine, and glucocorticoids (Fowden, 2003). Finally, IGFs play an essential role in bone metabolism. Interestingly, Akcakus *et al.* (2006) reported higher IGF-I levels in umbilical cord of LGA (large for gestational age) human neonates at delivery compared to SGA (small for gestational age) infants; in addition, the whole body bone mineral density was demonstrated to be higher in LGA neonates than in normal ones.

Insulin stimulates the fetal growth by increasing the mitotic rate and nutrient availability for the accretion of tissues. It minimally affects the tissue differentiation and maturation in utero, contrary to cortisol that keeps a critical role in differentiation and maturation of tissues and promotes the transition from fetal to adult modes of growth regulation, by inducing switch from IGF-II to IGF-I gene expression in fetal liver (Fowden, 1995). In canine species, as well as in humans, cortisol holds a critical role in fetal multi-organ maturation, above all in lung development; nevertheless, to the author knowledge, specific information concerning the adrenal glands development in canine fetus and the cortisol role in triggering for parturition are totally lacking. In the bitch, it was only reported that the plasmatic cortisol increases near to whelping, but there are no data about cortisol levels changes in fetal plasma during gestation (Veronesi, 2013).

Thyroxine affects both fetal tissue accretion and differentiation through a combination of metabolic and non-metabolic mechanisms, whereas the pituitary growth hormone is minimally involved in the control of fetal growth (Fowden, 1995).

Fetal hormones, therefore, promote growth and development in utero, by altering both metabolism and gene expression of fetal tissues (Fowden, 1995).

The interactions of the genome with the availability of oxygen and glucose, as well as the endocrine responses to changes in their supply, largely affect the fetal growth. Insulin and thyroid hormones are controlled by glucose and oxygen levels respectively, and they influence the fetal growth partially via IGF-I. Circulating IGFs are regulated by the glucose availability to the fetus. The materno-fetal transfer of substrates depends on the placental transfer capacity and placental utilization of those substances. The fetus checks the latter through its blood concentrations of oxygen and glucose, and possibly IGF-I. In the mother, placental hormones and proteins (progesterone, placental lactogen, placental growth factors) increase the circulating IGFs and alter both the stability and IGF-binding proteins levels. These changes may direct metabolic and growth adaptation of the mother to gestation, which promote an adequate transport of substrates to the developing fetus (Owens, 1991). Hormonal functions ensure that fetal growth rate is commensurate with the nutrient supply and that pre-partum maturation occurs in preparation for the extrauterine life (Fowden, 1995).

2.4.3 Fetal maturation

To the author knowledge, contrary to the other animal species, the availability of information about canine fetus development remains scarce. The fetal growth curves, only based on the ultrasonographic measurement of abdominal, cardiac, and biparietal diameter, were principally aimed to the prediction of the parturition date. Unfortunately, there are only few studies about the organic functional development; in most cases, the fetal heart rate was evaluated by repeated ultrasonographic observations. In this respect, it was reported that, during the second half of pregnancy, the fetal heart rate ranges from 170 to 260 beats for minute, with inter- and intra-fetus variability (Veronesi, 2013). Some studies performed on dog fetuses during the last 3 weeks of gestation in order to investigate the effect of hypoxia, induced by the compression of maternal abdominal aorta, on fetal heart rate, tissues, and plasmatic levels of pH, pO2, and pCO2, demonstrated that the notable decrease of fetal heart rate, few seconds after the beginning of the compression, represents an early signal of fetal hypoxia (Monhelt *et al.*, 1988). However, in both humans and other domestic animals, it was suggested that the correct interpretation of fetal heart rate changes would require continuous and prolonged recordings. Recently, the blood flow of uterine and umbilical arteries was evaluated from 44 days of

pregnancy to the parturition; in the future, this parameters could be useful for the diagnosis and monitoring of pathologic pregnancies (Veronesi, 2013).

2.5 Normal pregnancy

2.5.1 Physiological changes in the dam

Along gestation, the increased metabolic requirement implies some maternal physiological changes. Blood volume increases by 40% to compensate for the large amounts of blood and fluids lost at whelping. The volume increase is primarily formed by plasma, with a following haemodiluition (the hematocrit is 30-35% at term) (Smith, 2007; Linde Forsberg, 2010). An increase in cardiac output was documented, due to an enhanced heart rate and stroke volume (Linde Forsberg, 2010). Lùcio *et al.* (2009) investigated the peri-partum hemodynamic status of bitches with normal birth or dystocia, by evaluating the heart rate, systolic and diastolic blood pressure, and glucose level pre-partum, intra-partum, immediately after whelping, and after 1 hour. Heart rate was high in all cases, and blood pressure was generally normal; although systolic and diastolic blood pressures were highest during intra-partum stage and sometimes during the immediate post-partum phase, significant differences were not observed between groups. Blood glucose levels were always within the normal range, despite lower values in pre-partum period.

Since the cranial displacement of the diaphragm due to the pregnant uterus, the functional residual capacity of the lungs is decreased and oxygen consumption along gestation increases by 20%. Furthermore, pregnant bitches show delayed gastric emptying, as a consequence of a decreased gastric motility and stomach displacement (Linde Forsberg, 2010). During pregnancy, the physiological increase in progesterone levels induces the growth hormone (GH) secretion, that may cause the insulin resistance, above all in middle-aged and older bitches that are pregnant or were recently in oestrus. Additionally, a reduced production of glucose via gluconeogenesis, glycogenolysis, and lipolysis was proved. The condition of type 2 diabetes is usually limited to the gestational phase, nevertheless some bitches seldom develop a pre-partum hypoglycaemia, with following muscle weakness, convulsions or collapse (Linde Forsberg, 2010).

Towards the term of pregnancy, the mineralization of the fetal skeleton, lactation, and myometrial activity increase the need of calcium. The inappetence

and respiratory alkalosis from panting may reduce the availability of free calcium, leading to deficient secretion of parathyroid hormone (PTH), with a resulting decrease in blood calcium levels (Hollinshead *et al.*, 2010; Linde Forsberg, 2010).

Concerning the hormonal situation, increased concentrations of 15-keto-dihydroprostaglandin F2α (PGF2α) were documented 24-36 hours before whelping and again at the onset of parturition (Concannon *et al.*, 1989; Veronesi *et al.*, 2002; Verstegen-Onclin and Verstegen, 2008; Linde Forsberg, 2010), with consequent decrease of progesterone levels (Veronesi *et al.*, 2002). Furthermore, serum cortisol concentrations increase at delivery, remaining high for 12 hours and declining to basal values after 36 hours (Veronesi *et al.*, 2002; Linde Forsberg, 2010). Olsson *et al.* (2003) evaluated plasma levels of vasopressin, oxytocin, cortisol, and PGF2α metabolite during whelping, demonstrating that probably these hormones play different roles. Generally, all the hormonal concentrations appeared higher at birth of the first puppy. Vasopressin and cortisol levels remained high also at birth of the second puppy, then declined; oxytocin was high throughout parturition, whereas PGF2α metabolite until 1h after whelping. Plasma vasopressin concentrations were strongly correlated with cortisol, but less with PGF2α metabolite and not significantly with oxytocin. Baan *et al.* (2008) examined the hormonal changes in spontaneous and induced parturition in dogs. Based on these findings, PGF2α metabolite concentrations increased before whelping in both groups, with lower values in induced bitches; the metabolite levels reached a maximum in both groups during whelping and quickly decreased later, remaining elevated in induced group. In both groups cortisol reached similar maximum concentrations during the last 30 hours before the expulsion onset. During 3 days post-partum, cortisol was higher in induced group compared to spontaneous one. In both groups, estradiol-17-beta decreased, whereas prolactin increased between late gestational period and 30 hours before parturition.

Another study investigated the plasma oxytocin levels during late gestation and parturition in canine species, highlighting higher and more variable concentrations during the expulsive stage of whelping than during late pregnancy (Klarenbeek *et al.*, 2007).

2.5.2 Duration of canine pregnancy and prediction of the parturition date

The assessment of the gestational age and fetal maturation, as well as the prediction of parturition day, is of considerable clinical importance in the bitch to provide obstetrical assistance during spontaneous whelping, but especially in case of threatened abortion, prolonged gestation, preterm labor, previous history of dystocia, or elective cesarean section (Kim *et al.*, 2007; Lopate, 2008; Linde Forsberg, 2010; Veronesi, 2013). Since the gestation length in canine species is relatively short (only 63 days from ovulation) compared to other domestic species, the accuracy in prediction of the parturition date is absolutely indispensable to ensure the complete fetal maturity (Lopate, 2008; Veronesi, 2013). Therefore, fetuses are immature at birth; the most development of major organ systems occurs during the last days of gestation to guarantee the extrauterine survival, even if it continues for several weeks-months after birth. Additionally, since the type of canine placenta, the overcoming of the estimated parturition date by more than 2 days implies the need of more nutritional support, with following intrauterine fetal death. Thus, it is critical to ensure the achievement of the maximal gestational age, without overcome it, prior to delivery (Lopate, 2008).

The keys for timing the duration of canine pregnancy are both the preovulatory LH and the concomitant increase in serum progesterone concentrations, rather than the insemination date or estrus onset (Kutzler *et al.*, 2003; Kim *et al.*, 2007; Smith, 2007). In fact, many studies evidenced a minimal correlation between the onset of estrus and the ovulation timing; thus, it does not represent an accurate predictor of ovulation or parturition date. Since the extreme variability of the estrous cycle length and receptive behavior in the bitch, as well as the sperm lifespan in female reproductive tract, the breeding date is not an accurate method either to estimate the gestational age (Rendano *et al.*, 1984; Shille and Gontarek, 1985; Johnston *et al.*, 2001; Lopate, 2008). Furthermore, the pregnancy length could be more variable in case of multiple mating (Veronesi, 2013). However, the canine full-term pregnancy was reported to last 57-72 days from the insemination (Concannon *et al.*, 1983; Rendano, 1983; Rendano *et al.*, 1984; Johnston *et al.*, 2001; Kim *et al.*, 2007; Lopate, 2008; Linde Forsberg, 2010; Veronesi, 2013).

Fortunately, two breeding management methods, commonly used in clinical practice, can be adjusted to accurately predict the date of whelping: the

assessment of the ovulation timing, by serial measurements of serum progesterone concentrations, and transabdominal ultrasonography (Kim *et al.*, 2007).

The duration of canine gestation is 65±1 days from the preovulatory serum LH peak (Concannon *et al.*, 1983; Kim *et al.*, 2007; Linde Forsberg, 2010; Veronesi, 2013), which coincides with the initial sharp rise in serum progesterone concentrations to ≥1.5 ng/ml (Kutzler *et al.*, 2003; Linde Forsberg, 2010). It is recommended to perform serial preovulatory serum progesterone measurements to estimate the day of the LH peak (day 0), followed by transabdominal ultrasonography for the confirmation (Kutzler *et al.*, 2003; Kutzler *et al.*, 2003; Kim *et al.*, 2007). The preovulatory progesterone measurement was based on the fact that the ovulation occurs approximately 2 days after the serum LH peak (day 0) (Concannon *et al.*, 1983; Rendano *et al.*, 1984; Shille and Gontarek, 1985; Johnston *et al.*, 2001; Kim *et al.*, 2007; Lopate, 2008) and the coincident increase of progesterone levels represents the cheaper and easier tool to estimate day 0 and to plan the mating (Goodman, 2001; Kutzler *et al.*, 2003; Kim *et al.*, 2007). By analyzing the serum progesterone concentrations before mating, the accuracy of prediction date of parturition within an interval of ±1, ±2, and ±3 days was 67%, 90%, and 100%, respectively (Linde Forsberg, 2010).

Pregnancy may be diagnosed as early as 19-21 days, when the conceptuses are approximately 1 cm in diameter (Shille and Gontarek, 1985; England *et al.*, 1990; Nyland and Mattoon, 2002; Lopate, 2008). Fetal heartbeats and movement may be detected as early as day 23 (England *et al.*, 1990; Nyland and Mattoon, 2002; Lopate, 2008). Davidson and Baker (2009) reported that ultrasonography allows for the evaluation of early fetal cardiac motion at 21-22 days post LH peak, as well as fetal movements at 31-32 days, and also the fetal heart rate, enabling the assessment of viability. Several ultrasonographic fetal measurements seem to be useful to accurately predict the parturition date: among these, the embryonic vesicle diameter, crown-rump length, body diameter, and biparietal diameter are included (Kutzler *et al.*, 2003). At least two measurements were recommended on ≥2 fetuses (Kim *et al.*, 2007). Both the diameter of the inner chorionic cavity on day 18-37 following ovulation (Cartee and Rowles, 1984; Shille and Gontarek, 1985; England *et al.*, 1990; Yeager *et al.*, 1992; Luvoni and Grioni, 2000; Son *et al.*, 2001; Nyland and Mattoon, 2002; Beccaglia and Luvoni, 2006; Luvoni and Beccaglia, 2006; Lopate 2008) and the fetal head diameter on days 37-38 to

parturition showed the best correlation with gestational age and parturition date (Beccaglia and Luvoni, 2006; Lopate, 2008; Davidson and Baker, 2009; Linde Forsberg, 2010; Veronesi, 2013). It was demonstrated that these parameters are well correlated to the prediction of parturition date (±1 day), above all in small- and medium-sized bitches (Veronesi, 2013). Between 70 and 77% of whelping dates were predicted within 1 day based on the biparietal diameter and the inner chorionic cavity, respectively, whereas between 85 and 86% within 2 days (Beccaglia and Luvoni, 2006). In early to mid-pregnancy (<37-40 days), the use of the inner chorionic cavity was between 64 and 91% accurate (±1d) in both small and medium breeds, and between 85 and 88% accurate in large breeds (±2d) to estimate the day of parturition (Luvoni and Grioni, 2000; Son *et al.*, 2001; Beccaglia and Luvoni, 2006; Luvoni and Beccaglia, 2006; Levstein-Volanski, 2008; Lopate, 2008). In late pregnancy (>40days), the biparietal diameter is the most accurate measurement tool (Son *et al.*, 2001; Kutzler *et al.*, 2003; Luvoni and Beccaglia, 2006; Levstein-Volanski, 2008; Lopate, 2008). The accuracy of the biparietal diameter measurements within 1 day of actual parturition was 64-75% in small breeds and 65% in medium, and within 2 days this increased to 85-88% and 81-86%, respectively (Son *et al.*, 2001; Beccaglia and Luvoni, 2006; Luvoni and Beccaglia, 2006; Levstein-Volanski, 2008). The measurement formulas for medium-sized bitches could be used also for giant and toy breeds, if corrected for the extremes in size; specifically, it is recommended to subtract 2d for giant breed bitches (>40kg) and add 1d for small-breed bitches (<9kg), after gestational age was calculated (Kutzler *et al.*, 2003; Lopate, 2008). Finally, also the deep portion of fetal diencephalo-telencephalic vesicle, that can be visualized from days 35 to 58 as a symmetric anechoic area found on sagittal midline in fetal skull, was reported as another tool to determine the gestational age. It represents fetal thalamus and primordial basal nuclei (Beccaglia and Luvoni, 2004; Beccaglia *et al.*, 2008).

Ultrasonography and radiography are considered the best methods to assess the fetal maturation (Smith, 2007; Lopate, 2008). B-mode and four-dimensional colour Doppler ultrasonography were employed to assess the diameter of pregnancy structures, as well as fetal size, during gestation (Kutzler *et al.*, 2003; Linde Forsberg, 2010). Thus, this allows to confirm the pregnancy and to predict the parturition date, through the fetal measurements and the evaluation of the organs development progression, above all when the information about the

mating date and progesterone concentrations measurements are not available (England and Allen, 1990; Yeager *et al.*, 1992; Nyland and Mattoon, 2002; Levstein-Volanski, 2008; Lopate, 2008; Veronesi, 2013). The embryo, oblong and adjacent to the wall of the uterus, appears for the first time within the gestational sac by days 25 or 26. The heartbeat is first visible at 25-26d, whereas at days 27-28 the embryo is suspended by fetal membranes. The placenta can be detected as early as day 26-27 as a distinct structure lining the uterus; it becomes zonary by days 29-31, and the edges curl inward by days 32-34. The embryo is located dependently in the chorionic cavity by days 29-33. The bladder is first visible between 35 and 39 days; the stomach 36-39d; kidney and eyes 39-47d; intestine 57-63d. The peristalsis is evident between 62 and 64 d (England *et al.*, 1990; Yeager *et al.*, 1992; Nyland and Mattoon, 2002; Levstein-Volanski, 2008; Lopate, 2008). Other fetal structures used to time pregnancies by ultrasonography are fetal limb buds, first detectable on day 33-35; eyes, kidney, and liver on day 39-47; and intestine on day 57-63 (Linde Forsberg, 2010).

As the progesterone concentrations, the accuracy of parturition date estimation was not significantly affected by the litter size (Lopate, 2008). Some studies reported no litter size effect on the gestation length (Kutzler *et al.*, 2003; Luvoni and Beccaglia, 2006), whereas other suggested longer gestation in smaller litter size and shorter in larger litter size (Okkens *et al.*, 1993; Okkens *et al.*, 2001; Eilts *et al.*, 2005; Beccaglia and Luvoni, 2006; Bobic Gavrilovic *et al.*, 2007; Linde Forsberg, 2010). A recent study, performed on Drever bitches, proposed that each additional puppy above the average for the breed results in a shortening of pregnancy length by 0.25 days, and for each puppy less than the breed average a corresponding lengthening of pregnancy occurs (Bobic Gavrilovic *et al.*, 2007).

Some important differences in fetal growth rates in late gestation were correlated to the maternal body weight (Kutzler *et al.*, 2003; Kutzler *et al.*, 2003; Kim *et al.*, 2007). In previous studies, fetal growth was linear from days 17 to 30, subsequently became exponential (England, 1998; Kim *et al.*, 2007). After day 30, fetuses of small bitches (<9kg) grew slower, whereas fetuses of giant bitches (>40kg) grew faster, compared to medium and large breeds (Kutzler *et al.*, 2003; Kutzler *et al.*, 2003; Kim *et al.*, 2007). When corrected for the dam bodyweight, the overall accuracy for parturition date prediction by ultrasound method was 75% for the day 65±1d prediction, 87% for the day 65±2d prediction, and 100%

for the day 65±3d prediction (Kutzler *et al.*, 2003; Kutzler *et al.*, 2003; Kim *et al.*, 2007).

Furthermore, the gestation length likely depends on the breed (Linde Forsberg, 2010). In that regard, German Shepherds (Okkens *et al.*, 1993; Okkens *et al.,* 2001) and Hound dogs (Eilts *et al.*, 2005) seem to have a shorter gestation length, whereas West Highland White Terrier dogs a longer one (Okkens *et al.*, 1993; Okkens *et al.,* 2001).

Radiographic approach can help to estimate the gestational age and the number of fetuses, but not to determine fetal readiness for birth, because there is some overlap of radiographic detail. The fetus may be completely mineralized as early as 58 days after LH surge, but at this stage it would not survive ex-utero (Lopate, 2008). By radiography, the fetal skeleton is rarely visible before day 42 (Linde Forsberg, 2010). The structures more commonly employed to determine the stage of pregnancy are the following: the skull on day 45-49 after LH peak; scapula, humerus, and femur 46-51 days; radius, ulna, and tibia 50-53 days; pelvic bones and ribs on day 53-59; coccygeal vertebrae, fibula, calcaneus, distal extremities 55-64 day after LH peak; teeth on day 58-63 (Rendano, 1983; Rendano *et al.*, 1984; Toal *et al.*, 1986; Johnston *et al.*, 2001; Lopate, 2008).

CHAPTER 3

Parturition in canine species

3. Parturition in canine species

3.1 Endocrine and mechanical events

In mammals, at the end of gestation, the fetus promotes a cascade of complex endocrine/biochemical events, working as the trigger for the onset of parturition. The fetal hypothalamic-pituitary-adrenal (HPA) axis is mandatory for the beginning of delivery. Towards the term of gestation, the available intrauterine space becomes limited for fetus, causing fetal stress. Such stress induces the release of adrenal corticotrophin (ACTH) by the fetal anterior pituitary, that stimulates the fetal adrenal cortex to release corticoids. The increase of fetal corticoids induces a cascade of events which change dramatically the endocrine condition of the dam (Senger, 2003). Although the fetus was often considered as the trigger for parturition, to the author knowledge this remains still unproven in canine species (Veronesi, 2013). In dogs, it is generally believed that stress, caused by the reduction in the placental nutritional supply, stimulates the fetal HPA axis, resulting in the release of adrenocorticosteroid hormone, considered the trigger for parturition (Linde Forsberg, 2010). In mammals, these endocrine variations lead to the removal of the myometrial "progesterone block", allowing for the onset of myometrial contractions, and to the increase of reproductive tract secretions, especially by the cervix. The first event occurs due to the conversion of progesterone into estradiol by some enzymes induced by fetal cortisol; this marks the beginning of the first stage of whelping. In addition, fetal corticoids promote the placental synthesis of PGF2α, which is involved in the removal of the "progesterone block" (Senger, 2003). Even in the bitch, it was documented that the progesterone decreases in maternal plasma, while the PGF2α metabolites increase, before the parturition, but the activation mechanisms of these hormonal changes have to be clarified (Veronesi, 2013). In canine species, the increase in both fetal and maternal cortisol is thought to stimulate the release of PGF2α from the fetoplacental tissue, with consequent plasma progesterone concentrations decline. Kowalewski *et al.* (2010) showed the pre-partum increase of PGF2α as a consequence of a strong up-regulation of PTGS2 (cyclooxygenase 2, COX2) in the fetal trophoblast with the withdrawal of progesterone having a signaling function (Linde Forsberg, 2010). In mammals, as both estradiol and prostaglandin become elevated, the myometrium begins to display noticeable contractions. The increase of estradiol and PGF2α

levels, associated with the simultaneous regression of corpus luteum and progesterone decline, creates the ideal condition for the onset of the uterine contractions. The dilation of the cervix and fetal entry into the cervical canal promote the first stage of parturition (Senger, 2003). In the bitch, concurrently with the gradual decrease in plasma progesterone levels before whelping, a progressive qualitative change was documented in uterine electrical activity; specifically, a significant increase in uterine activity occurs during the last 24 hours before parturition, with the final decrease in plasma progesterone concentration to below 2 ng/ml. Probably, the change in the oestrogen:progesterone concentrations ratio causes the placental separation and cervical dilation in dogs, despite the oestrogen increase was not detected before whelping, contrary to many other species (Linde Forsberg, 2010). In mammals, the high pressure on the cervix, guaranteed by myometrial contractions as well as fetal movements, stimulates cervical pressure-sensitive neurons which synapse with hypothalamic oxytocin-producing neurons. Oxytocin, released into the systemic circulation from the posterior pituitary lobe, facilitates the myometrial contractility induced previously by estradiol and PGF2α. While the pressure against the cervix is increasing, the oxytocin secretion and thus the strength of myometrial contractions reach the peak, so that the fetus enters the cervical canal and the first stage of parturition is complete (Senger, 2003). In canine species, only few studies investigated oxytocin plasmatic levels in the bitch during gestation and whelping, evidencing higher and more variable plasmatic concentrations during the expulsive phase than during pregnancy. However to date, the relationship between the oxytocin secretion and the myometrial contractility was not still elucidated. It was only reported that the bitches affected by dystocia showed lower plasmatic levels of oxytocin, vasopressin, and PGF2α metabolite, compared to normal subjects (Bergström *et al.*, 2010; Veronesi, 2013).

Relaxin is another important hormone involved in successful whelping. It is a glycoprotein produced by the corpus luteum or placenta, depending on species (Senger, 2003). In the pregnant bitch, this hormone is produced primarily by the placenta, even if it was also detected in the ovaries and uterus (Linde Forsberg, 2010). In mammals, the relaxin synthesis is stimulated by PGF2α and promotes the softening of the cervical connective tissue and the elasticity of the pelvic ligaments, preparing the birth canal to the fetal passage (Senger, 2003; Linde

Forsberg, 2010). In canine species, the role of the relaxin in the functional regulation of the cervix and myometrium remains to clarify, despite high plasmatic levels were detected in the bitch during late gestation and parturition (Veronesi, 2013). Relaxin concentrations increase gradually along the last two-thirds of pregnancy and it usually decline abruptly after whelping, even if they can be detected for up to 9 weeks post-partum (Linde Forsberg, 2010).

Among the dramatic effects of estradiol increase before parturition, the onset of the secretory activity of the female reproductive tract is included. Both the cervix and vagina start to produce mucus, which washes out the cervical seal of pregnancy and lubricates the birth canal. While myometrial contractions increase, the fetus begins to press on the fetal membranes, leading to their rupture, with subsequent loss of amniotic and allantoic fluids. During the passage through the birth canal, the fetus becomes hypoxic and starts to move, promoting in turn further myometrial contractions. The uterine contractions, associated to abdominal muscle contractions of the dam, lead to the fetal expulsion, which marks the second stage of whelping.

In most species, the expulsion of fetal membranes quickly follows the fetal expulsion and it characterizes the third stage of parturition. Expulsion of the fetal membranes requires the dislodgement of the chorionic villi from the crypts of the maternal side of placenta, which is believed to be induced by powerful vasoconstriction of arteries in the villi (Senger, 2003).

3.2 Stages of whelping

Several days before parturition, the bitch may become restless, secluded or excessively attentive, and anorexic. She may exhibit nesting behavior 12-24 hours before whelping, likely due to the uterine contractions increase. In primiparous bitches, lactation may be noted less than 24 hours before parturition, whereas after several pregnancies colostrum can be detected as early as 1 week before whelping (Linde Forsberg, 2010; Veronesi, 2013). The decline in rectal temperature, due to the final abrupt decrease in progesterone concentrations, represents the most consistent change (Veronesi *et al.*, 2002; Smith, 2007; Linde Forsberg, 2010; Rickard, 2011; Veronesi, 2013). During the last week of canine gestation, the rectal temperature fluctuates and finally declines sharply 8-24 hours before whelping. Approximately 10-14 hours later, the plasma progesterone level is lower than 2 ng/ml (Smith, 2007; Linde Forsberg, 2010;

Veronesi, 2013). This hormone is thought to be thermogenic, thus a decrease in rectal temperature follows the progesterone decline. After that, the rectal temperature rises again and may become higher than normal (Linde Forsberg, 2010; Veronesi, 2013). To assess the pre-partum decrease in body temperature properly, measurements should start at least two weeks before the predicted parturition date and made twice a day (Veronesi *et al.*, 2002; Veronesi, 2013); subsequently, every 1-2 hours while the temperature is decreasing, and less frequently when the temperature is increasing again (Veronesi *et al.*, 2002; Linde Forsberg, 2010). The rectal temperature in miniature-sized breeds can fall to 35°C, in medium-sized bitches to around 36°C, whereas it seldom falls below 37°C in bitches of giant breeds. This difference is probably due to the different ratio between surface area and body volume; the hair coat may also have an influence (Linde Forsberg, 2010).

The relaxation of the pelvic and abdominal musculature, as well as of the perineal region, that occurs before whelping due to relaxin, is a consistent but subtle indicator of impending parturition (Linde Forsberg, 2010).

The first stage of parturition usually lasts 6-12 hours, but it may be 24 or 36 hours long, especially in a nervous primiparous females (Senger, 2003; Linde Forsberg, 2010; Rickard, 2011). This phase is characterized by vaginal relaxation, cervical dilation, and intermittent uterine contractions, without abdominal straining. Restless behavior, panting, tearing up, rearranging of bedding, shivering, anorexia, and occasionally vomiting may be noted (Linde Forsberg, 2010; Rickard, 2011). The propulsive efforts of the uterus push the fetal membranes ahead of the fetus, promoting the cervix dilation. During pregnancy, the fetuses are oriented 50% caudally and 50% cranially within the uterus, but during the first stage of whelping they rotate on their long axis, resulting in 60-70% of puppies in anterior and 30-40% in posterior presentation (Linde Forsberg, 2010).

The duration of the second stage of whelping is usually 3-12 hours, seldom 24 hours (Senger, 2003; Linde Forsberg, 2010; Rickard, 2011). During this phase, the rectal temperature rises to normal or slightly above normal. Internally, the first fetus moves toward the pelvis, and the subsequent uterine contractions are coupled to abdominal straining. As the fetus enters the birth canal, the allantochorionic membrane may rupture and a clear discharge may be observed

(Linde Forsberg, 2010; Rickard, 2011). The first fetus is usually delivered, covered by the amniotic membrane, within 4 hours since the onset of the second stage. The interval between births ranges between 5 minutes and 2 hours in normal uncomplicated whelping. In almost 80% of cases, fetuses are delivered alternatively from the two uterine horns (Linde Forsberg, 2010).

The third stage of canine parturition usually occurs immediately after the fetal expulsion, but it can occurs 15 minutes after the delivery of each fetus (Senger, 2003; Linde Forsberg, 2010; Rickard, 2011). However, more fetuses may be born before the passage of their placenta. The bitch normally eats the placenta. Lochia (the greenish post-partum discharge of fetal fluids and placental remains) may be detected for up to 3 weeks or even more. Normally uterine involution is complete after 12-15 weeks (Linde Forsberg, 2010).

CHAPTER 4

Preparation for birth

and

neonatal adaptation

4. Preparation for birth and neonatal adaptation

4.1 Hypothalamic-pituitary-adrenal axis

4.1.1 Modulation

In most animal species, birth is triggered by the activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis; consequently, during late gestation, an enhance in fetal plasma glucocorticoids was reported (Challis *et al.*, 2000; Challis *et al.*, 2001; Schwartz and McMillen, 2001). The timing of HPA axis activation depends on species and it is still unknown in carnivores (Vannucchi *et al.*, 2012). Cortisol is a corticosteroid hormone, that is synthesized by the adrenal glands under the regulation of the anterior pituitary adrenocorticotropic hormone (ACTH) which, in turn, is stimulated by the corticotrophin-releasing hormone (CRH), secreted by the hypothalamic paraventricular nucleus. Corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) work as the principal modulators of HPA axis (Mastorakos and Illas, 2003). Corticotrophin-releasing hormone is mainly produced by hypothalamus, even if it was detected in theca and stroma cells, as well as in corpus luteum cells, of human and rat ovaries. Furthermore, even the cytoplasm of the endometrial glandular cells contains CRH, and myometrium shows receptors for this hormone. Both fetal and maternal CRH may regulate the implantation process, by acting on the cytotrophoblast cells. Along human gestation, maternal plasma CRH levels rise exponentially from the first trimester of pregnancy, due to the placenta and fetal membranes production. The presence of CRH-binding protein, that restricts the bioactivity of circulating CRH, was documented only in plasma and amniotic fluid of human beings. Maternal secretion of pituitary ACTH, as well as plasma ACTH concentrations, increase during gestation, paralleling to the rise of plasma cortisol levels, since pregnancy is a physiologic period of hypercortisolism. CRH was assessed in fetal hypothalamus by 12th week of gestation and its fetal plasma concentrations appeared 50% less than those in maternal plasma, due to the slower placental secretion in fetal compartment. Also ACTH can be detected in fetal plasma at 12 weeks pregnancy, with higher levels before 34 weeks. Although the increased adrenal steroid synthesis in fetus, the cortisol of maternal origin predominates in fetal circulation, at least in non human primates. Fetal adrenal glands synthesize cortisol from the progesterone supplied by placenta, even if another source is

represented by the amniotic fluid, where cortisol is converted from cortisone by the choriodecidua. In humans, maternal plasma CRH, ACTH, and cortisol levels rise during normal delivery and decline after 4 days, without any correlation between maternal ACTH and cortisol concentrations at this stage. In sheep, placental CRH promotes the fetal production of ACTH, which in turn leads to a peak of fetal cortisol secretion that triggers parturition. During post-partum period, HPA axis generally recovers from its activation during gestation (Mastorakos and Illas, 2003).

Arginine-vasopressin (AVP) was assessed in fetal hypothalamus; it is usually detectable in human fetal neurohypophysis at 11 to 12 weeks of pregnancy and strongly increases over the next 12 to 16 weeks. The role of AVP is unclear, despite parturition seems to promote its fetal release (Mastorakos and Illas, 2003).

4.1.2 Glucocorticoid secretion

Glucocorticoids are involved in fetal maturation, regulation of immune response, and several physiological changes correlated with gestation (Gonzales *et al.*, 1986; Whittle *et al.*, 2001; Myatt and Sun, 2010). Studies performed on animals suggested a critical role of fetal cortisol even in the initiation of parturition (Challis *et al.*, 2000; Whittle *et al.*, 2001; Jenkin and Young, 2004; Myatt and Sun, 2010). In sheep, it was demonstrated that the maturing HPA axis leads to the release of increasing amount of cortisol towards the end of pregnancy. At placental level, this hormone induces the expression of some enzymes aimed to the synthesis of both estrogen and prostaglandin, which may stimulate uterine contractions (Anderson *et al.*, 1975; Flint *et al.*, 1978; Ma *et al.*, 1999; Myatt and Sun, 2010). Specifically, cortisol is able to stimulate the $P_{450}\,C_{17}$ hydroxylase, that produces estrogen from progesterone and prostaglandin synthases 2 (PGHS-2), essential for prostaglandin synthesis (Wu *et al.*, 2001; Myatt and Sun, 2010). In humans, the estrogen synthesis depends on the precursor dehydroepiandrosterone sulfate (DHEAS) because of the lack of $P_{450}\,C_{17}$ in placenta, despite the ability of cortisol to up-regulate the expression of prostaglandin synthesizing enzymes and the CRH synthesis in placenta and fetal membranes (McLean *et al.*, 1995; Karalis *et al.*, 1996; Blumenstein *et al.*, 2000; Challis *et al.*, 2000; Cheng *et al.*, 2000). Thus, both progesterone and estrogen levels rise with gestational age in women, although the progressive increase of

glucocorticoid concentrations in maternal and fetal circulation, as well as in the amniotic fluid (Casey *et al.*, 1985; Challis *et al.*, 2000; Myatt and Sun, 2010). Dehydroepiandrosterone and DHEAS are mainly produced by human fetal adrenal glands along pregnancy, because the enzyme 3beta-hydroxysteroid dehydrogenase (3β-HSD), responsible for the cortisol synthesis from cholesterol, is expressed in fetal adrenal glands since the last trimester of gestation (Mesiano and Jaffe, 1997; Myatt and Sun, 2010). Successively, the 3β-HSD expression leads to the cortisol synthesis by the human fetal adrenal glands (Ohrlander *et al.*, 1976; Mesiano and Jaffe, 1997). In comparison to other animals, the increase of the fetal cortisol concentration appears slower and less extent at the end of pregnancy, contrary to the hormonal level in maternal circulation and amniotic fluid, that rise dramatically (Blankstein *et al.*, 1980; Myatt and Sun, 2010). Since it is well known that high concentrations of circulating glucocorticoids are teratogenic for the growing fetus, the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) ensures low cortisol levels in fetal circulation, by converting cortisol into biologically inactive cortisone in placenta and fetal tissues (Shams *et al.*, 1998; Seckl *et al.*, 2000; Myatt and Sun, 2010). In humans, the expression of 11β-HSD2 starts early in gestation as a self-protective mechanism, so that the biologically inactive cortisone represents the major glucocorticoid hormone in fetal circulation (Murphy, 1981; Stewart *et al.*, 1994; Myatt and Sun, 2010). However, the glucocorticoids amount increase occurs at term of pregnancy in fetal circulation, since their critical role in fetal organ maturation and parturition. In this respect, several factors appear involved, such as weakened placental glucocorticoid barrier, scarce 11β-HSD2 or increased 11β-HSD1 expression, and increased cortisol synthesis (Ohrlander *et al.*, 1976; Tanswell *et al.*, 1977; Seron-Ferre *et al.*, 1978; Murphy, 1981; Giannopoulos *et al.*, 1982; Diaz *et al.*, 1998; Alfaidy *et al.*, 2003; Myatt and Sun, 2010). It was evidenced that both the expression and activity of 11β-HSD1 in fetal membranes rise with gestational age, paralleling to the cortisol levels in the amniotic fluid and fetal circulation, as this enzyme regenerates cortisol from cortisone (Tanswell *et al.*, 1977; Blankstein *et al.*, 1980; Alfaidy *et al.*, 2003; Myatt and Sun, 2010). Cortisol/cortisone ratio increases in amniotic fluid as gestation progresses, with higher values than in cord serum, suggesting fetal membranes as another source for cortisol secretion during pregnancy (Blankstein *et al.*, 1980; Myatt and Sun, 2010). Interestingly, this ratio appears significantly lower in the amniotic fluid of infants who develop

respiratory distress syndrome, proving the importance of cortisol source in fetal lung maturation (Smith *et al.*, 1977).

4.1.3 Cortisol functions

Multiple endocrine factors play an essential role in the transition to extrauterine life, and particularly in the regulation of the lung development, in several species, including rodents, primates, and humans (Bolt *et al.*, 2001). Specifically, the physiological increase of endogenous cortisol concentrations, some days before parturition, is of outmost importance for fetal final maturation, since in humans high cortisol levels in fetal circulation are strongly correlated to the structural and functional lung maturation (Bonanno and Wapner, 2009; Vannucchi *et al.*, 2012). At this regard, several events occur, such as the surfactant protein and phospholipid production, cellular maturation and differentiation, changes in interstitial tissue components, and regulation of the pulmonary fluids metabolism (Bolt *et al.*, 2001; Vannucchi *et al.*, 2012). In human medicine, the production of pulmonary surfactant by type II pneumocytes may be reliably correlated with the degree of fetal lung maturity and readiness for extrauterine life (Vestweber, 1997; Vannucchi *et al.*, 2012). Scarce surfactant synthesis, due to pulmonary immaturity, represents the main cause of respiratory distress syndrome, one of the most significant pathological conditions of fetal immaturity (Miyoshi *et al.*, 1998; Vannucchi *et al.*, 2012). To date, antenatal corticosteroids administration to pregnant women is considered a routine treatment to artificially induce pulmonary fetal maturation and significantly decrease the incidence of respiratory distress syndrome in premature newborns (Peltoniemi *et al.*, 2007; Vannucchi *et al.*, 2012). Even in ovine fetus it was documented that the antenatal steroids exposure improves the adaptation after birth by accelerating both parenchymal and vascular lung maturation (Houfflin-Debarge *et al.*, 2005).

Since the effects of glucocorticoids on both fetal canine lung and endogenous serum cortisol concentrations were not clearly delineated, Vannucchi *et al.* (2012) investigated this topic, suggesting that, despite the down-regulation on the HPA axis and the induction of premature whelping, maternal betamethasone treatment was able to provide similar vitality to the newborns puppies born by cesarean section at 58 days after ovulation when compared to the untreated neonates born at term. Additionally, Regazzi (2011) documented a structural

pulmonary maturation simultaneously to an improvement on neonatal clinical conditions in response to canine prenatal betamethasone administration.

The crucial role of cortisol in regulating surfactant synthesis in fetal lung was widely described in both humans and animals (Snyder *et al.*, 1981; Gonzales *et al.*, 1986; Mendelson *et al.*, 1986; Myatt and Sun, 2010). During the intrauterine development, the type II alveolar cells synthesize surfactant, intermittently discharged into the amniotic fluid (Snyder *et al.*, 1988; Van Golde *et al.*, 1988; Pryhuber *et al.*, 1991; Myatt and Sun, 2010). Surfactant is a particular mixture formed by phospholipids, nonpolar lipids, and proteins. Surfactant protein A (SP-A) is one of the most plentiful apoproteins specifically associated with pulmonary surfactant (Floros and Phelps, 1997) and its concentration in amniotic fluid increases abruptly along the third trimester of human pregnancy (Snyder *et al.*, 1988; Pryhuber *et al.*, 1991; Myatt and Sun, 2010). The surfactant phospholipids provide a source of arachidonic acid, useful for prostaglandin synthesis by amnion (Lopez Bernal *et al.*, 1989; Newman *et al.*, 1993) and it was documented that SP-A is actively involved in the regulation of immune function in fetal lung (Crouch and Wright, 2001). Recently, Condon *et al.* (2004) evidenced that SP-A injection into the amniotic fluid caused preterm labor in mouse, suggesting that prostaglandin-synthesizing enzymes in fetal membranes could be possible targets for this apoprotein. In fetal membranes, the SP-A expression seems to be stimulated by cortisol within the physiological range reached in the amniotic fluid during late gestation (Sun *et al.*, 2006a). Based on previous findings, it was speculated that both SP-A originated from fetal lung via amniotic fluid and SP-A synthesized locally in fetal membranes may participate in the onset of labor, by inducing the prostaglandin synthesis at term of pregnancy, which may parallel increase the expression of 11β-HSD1 in fetal membranes and cortisol in the amniotic fluid from the third trimester of gestation ongoing (Gibb, 1998; Sun *et al.*, 2006a; Myatt and Sun, 2010). Accumulating evidences indicated that this positive feedback loop would promote the fetal organ maturation and the trigger of parturition in humans, by ensuring abundant biologically active glucocorticoids and prostaglandin in fetal membranes or in amniotic fluid (Myatt and Sun, 2010).

Preterm birth could be due to a precocious activation of fetal HPA axis, reflecting a normal fetal response to an adverse intrauterine environment. Hypothalamic-pituitary-adrenal axis development is associated with a gradual

increase of ACTH and adrenal corticosteroids (cortisol in sheep and humans) in the fetal circulation during the last days of pregnancy, beyond an enhanced expression of mRNA encoding CRH in hypothalamus, proopiomelanocortin (POMC) in pituitary gland, and ACTH receptors and steroidogenic enzymes in fetal adrenal (Challis *et al.*, 2000; Challis *et al.*, 2001). At term, high levels of cortisol act on placenta/trophoblast derived cells to promote the expression of prostaglandin synthase type II (PGHS-II). In human gestation, cortisol also decreases the expression of 15-hydroxyprostaglandin dehydrogenase (PGDH) in chorionic trophoblast cells. Towards term of pregnancy, the increased synthesis and decreased metabolism lead to an enhanced output of primary prostaglandin, which in turn increases the activity of 11beta-hydroxysteroid dehydrogenase in human fetal membranes. Increased chorionic 11betaHSD-1 results in the rise of local generation of cortisol from cortisone, with further paracrine/autocrine stimulation of prostaglandin output. Increased fetal cortisol contributes to the maturation of the organ systems required for postnatal extrauterine survival (Challis *et al.*, 2001).

Although the specific mechanisms initiating parturition can be different among animal species, glucocorticoids were proposed as the factors synchronizing the fetal maturation with the trigger of labor in nearly all species investigated (Jenkin and Young, 2004; Myatt and Sun, 2010). In humans, chorioamnionitis is the most common type of infection and it was recognized as the leading cause for preterm labor. The membranes infection causes the activation of macrophages, which release proinflammatory cytokines, such as IL-1b and TNFα, and activate local stromal cells that in turn release proinflammatory cytokines (Smaill, 1996). Both IL-1b and TNFα stimulate prostaglandin synthesis in fetal membranes, as well as increase the estrogen and CRH production in placenta (Petraglia *et al.*, 1990; Nestler, 1993). Furthermore, it was proved that IL-1b and TNFα induce 11betaHSD-1 expression in ovary, kidney, adipose tissue, and osteoblast cells (Escher *et al.*, 1997; Cooper *et al.*, 2001; Tomlinson *et al.*, 2001; Yong *et al.*, 2002), but also in fibroblast cells and trophoblast cells derived from human fetal membranes (Sun and Myatt, 2003; Li *et al.*, 2006). The increased regeneration of cortisol might control the inflammation, but also promote fetal organ maturation and initiate parturition, to protect the fetus from deteriorating detrimental effects of infection.

Similar changes occur in case of fetal hypoxemia, that decreases the fetal pituitary ACTH response but increases fetal adrenal responsiveness. Excessive levels of feto-placental glucocorticoids, derived from maternal administration of synthetic corticosteroids or strong endogenous fetal cortisol production, result in the intrauterine growth retardation (IUGR). Therefore, fetuses exposed to high maternal glucocorticoids concentrations in late pregnancy are growth restricted with altered postnatal HPA responsiveness and insulin resistance of type 2 diabetes. In conclusion, the fetal HPA activity is crucial not only for determining the gestational length, but even to predict pathophysiologic adjustments in later life (Challis *et al.*, 2000; Challis *et al.*, 2001).

4.2 Effects of parturition on the newborn viability

More than 65% of mortality in puppies occurs at parturition or during the first week of age (Linde Forsberg, 2010). Despite the elevated perinatal loss rates and the clear effect of whelping on the neonatal mortality (Davidson, 2003; Indrebø *et al.*, 2007; Veronesi, 2013), to date only scarce information are available about the normal and pathologic clinical course of whelping (Veronesi, 2013). During the first two weeks of age, essential adaptations to countless factors have to take place simultaneously with the development of vital functions that were not performed during intrauterine life. For instance, pulmonary respiration develops to guarantee the efficiency of gas exchange, known previously as a placental activity. Therefore, the neonatal period is critical, so that it is crucial to study the typical progression of the newborn adaptation to improve the puppies management (Vannucchi *et al.*, 2012).

The successful immediate adaptation to the extrauterine life depends on morphological, physiological, and biochemical maturation of the pulmonary parenchyma (Vannucchi *et al.*, 2012). Normally at birth, humans and most domestic animals undergo a short period of fetal asphyxia due to the uterine contractions, which might result in neonatal transient hypercapnia and acidaemia (Ruth and Raivio, 1988). At the onset of labor, the hypoxia stimulates fetal maturation, by inducing the production of pulmonary surfactant (Martin and Crump, 2003). To date, only limited data concerning blood gas parameters in canine newborns are available (Lúcio *et al.*, 2009), nevertheless the necropsy performed on several newborn puppies, dead intra-partum or early after birth, supported the role of neonatal asphyxia as the main cause of death (Linde

Forsberg, 2010; Veronesi, 2013). As in other species, the oxygenation of the canine fetus during delivery is threatened by the reduction in the uterine blood flow, as well as in the maternal part of placenta, the detachment of the zonary placenta, and the traction on the umbilical cord. Based on some observations during cesarean sections, it was presumed that the placenta remains attached to the uterine wall until shortly before or shortly after the fetal entry into the pelvic cavity; however, the exact moment of its total detachment was not still understood (van der Weiden *et al.*, 1981; Veronesi, 2013). The umbilical cord of the newborn puppies is short, so probably it undergoes traction during the fetal advancement in the birth canal, with following vasoconstriction of the umbilical vessels.

The adjustment of all the new vital functions, such as spontaneous respiration, adaptation of cardiovascular system, development of specific metabolic functions, redirecting of bloodstream, acquisition of muscular tonus, and sensitization to external stimuli, require some times (Crissiuma *et al.*, 2005). Thus, a depression of vital functions should be considered normal immediately after birth, in response to the transition between fetal and extrauterine life (Lúcio *et al.*, 2009). Based on the respiratory system examination, 78% of eutocic canine neonates exhibit irregular respiration associated with mild/moderate breath sounds immediately at birth, that decreases to 28% within 5 minutes (Silva *et al.*, 2009). Uterine contractions during whelping cause a physiological reduction in blood flow from mother to fetus, decreasing partially the oxygen pressure in fetal blood. When hypoxia is severe or long lasting, it produces a significant pO_2 reduction and reflex cerebral vasodilatation, combined with peripheral vasoconstriction (Siristatidis *et al.*, 2004). Indeed, it is not unexpected that, also in normal spontaneous delivery, a moderate or severe combined respiratory-metabolic acidosis occurs in almost every newborn puppy, as the pulmonary expansion starts gradually and does not allow gas exchange to reach immediate balance (van der Weiden *et al.*, 1989; Lúcio *et al.*, 2009; Veronesi, 2013). Lúcio *et al.* (2009) reported that, one hour after birth, puppies still exhibited acidosis because, despite a significant increase, pH values remained below the reference values. This period is not sufficient to achieve full recovery, although a considerable improvement thanks to tachypnoic response. Moreover, physiological hypoxia and increase in neonatal metabolism favour anaerobiosis, which maintains the state of metabolic acidosis. The fast pCO_2 decrease during

the first 15 minutes after birth proved that the respiration balances the respiratory acidosis very quickly. The most newborn puppies, included those affected by severe acidosis, reaches a normal acid-base balance within 3 hours after birth (van der Weiden *et al.*, 1989). The high glycogen levels, stored in fetal tissues during late pregnancy, and the transient body temperature reduction after birth represent the main factors responsible for the neonatal resistance against intra-partum asphyxia (Veronesi, 2013).

In humans, main disorders during neonatal period are secondary to hypoxia (Siristatidis *et al.*, 2004). In pigs, prolonged or intermittent asphyxia in utero or during labor decreases the newborns viability and reduces the adaptation ability to the extrauterine life (Herpin *et al.*, 1996). Canine newborns born by dystocic labor exhibit higher degrees of depression at birth than those born by normal whelping (Lúcio *et al.*, 2009). Particularly, Silva *et al.* (2009) observed irregular respiratory pattern in 87% of cases at birth and in 62% of subjects after 5 minutes. Regarding the newborn puppies born by cesarean section, respiratory rate at 60 minutes of life was lower compared to the other ones; additionally, respiratory alterations were found in 70% of cases at birth and in 45% of subjects after 5 minutes. Probably when the cesarean section is performed before the physiological onset of whelping, the mechanisms responsible for final pulmonary maturation are not appropriately activated. Consequently, the lack of stimuli from compression along the vaginal canal in neonates born by cesarean section reduces reflex respiration (Vannucchi *et al.*, 2012).

The Apgar scoring system is an easy and reliable method for evaluating both human and animal neonates. Nevertheless, its use is not yet widespread in veterinary medicine. A recent study performed by Veronesi *et al.* (2009) assessed a modified Apgar scoring system for routine evaluation of newborn puppies, based on the detection of heart rate, respiratory effort, reflex irritability, motility, and mucus color. Specifically, Apgar score at 5 minutes after birth was proved to be a good indicator of the newborn viability and short-term survival prognosis.

4.3 Fetal distress

The term "fetal distress" indicates a fetal well-being compromise, likely due to an unsuitable uterine environment. Cardiotocography remains the cornerstone for the diagnosis of fetal distress, despite its high sensitivity and low specificity (Vintzileos *et al.*, 1995; Tharmaratnam, 2000; Wiberg-Itzel *et al.* 2008; Heinis *et*

al., 2011). This technique reduced the intra-partum fetal mortality but not long-term neonatal morbidity or incidence of cerebral palsy. Obviously, the diagnosis is simple when there are clear signs of fetal compromise, such as late decelerations in presence of IUGR and oligohydramnios. Unfortunately, the signs of fetal compromise are often subtle and not so easily observable; among these, a meconium change in the amniotic fluid, rising base-line fetal heart rate, absent accelerations, atypical variable decelerations, or a combination of more findings are included (Tharmaratnam, 2000). Monhelt *et al.* (1988) recorded in dog the fetal heart rate, transcutaneous pO_2 and pCO_2, and tissue pH during hypoxic episodes obtained by blocking the maternal abdominal aorta. Thus, the late deceleration of fetal heart rate represents an early sign of fetal hypoxia, whereas the fetal blood pH decrease beyond that level normally seen during labor occurs later.

Since the heart tracings often were not reassuring but only few fetuses were really hypoxic, a diagnostic test seemed to be necessary. In 1962 the sampling of blood from the fetus scalp was introduced during labor to analyze pH values (Bretscher and Saling, 1967). This technique was regarded the ideal tool for the identification of intra-partum fetal hypoxia and, to date, it represents a diagnostic test in cases with suspicious cardiotocographic findings. Arbitrarily, a pH <7.20 was chosen as cut-off value to recommend intervention. However, the pH analysis is complicated, needs a relatively large amount of blood (30-50 μl), and sampling failure rates of 11-20% were reported (Westgren *et al.*, 1998; Tuffnell *et al.*, 2006). Moreover, it does not discriminate between respiratory and metabolic acidaemia, the latter associated with poor neonatal outcome (Goldaber *et al.*, 1991; Low *et al.*, 1994; MacLennan, 1999; Heinis *et al.*, 2011). For this reason, full blood gas analysis is considered better than pH alone (Low *et al.*, 1994; Low *et al.*, 1997). It was documented that lactate increases gradually as the metabolic acidosis progresses and represents a better marker than pH for immediate intervention, since it rises in subcutaneous tissue before pH decreases (Heinis *et al.*, 2011; Holzmann *et al.*, 2011). Lactate is a typical metabolite in anaerobic metabolism and reflects tissue hypoxia (Wiberg-Itzel *et al.*, 2008; Rubak and Henriksen, 2010; Holzmann *et al.*, 2011). Studies performed on the umbilical cord blood demonstrated a correlation between high lactate levels and fetal metabolism for anaerobic glycolysis, which takes place in fetal oxygen-deprived tissues. This finding may be of clinical importance when fetal distress or fetal

hypoxemia is caused by perinatal events (Borruto *et al.*, 2006). Interestingly, animal and human studies established that fetal scalp blood lactate levels adequately represent lactate concentrations in the fetal circulation and that fetal lactate production is endogenous (Kastendieck *et al.*, 1988; Nordström *et al.*, 1996). Recently, lactate was judged a good predictor of severe neonatal morbidity and intra-partum surveillance (Kruger *et al.*, 1999; Wiberg-Itzel *et al.*, 2008; East *et al.*, 2010; Wiberg *et al.*, 2010; Holzmann *et al.*, 2011); thus, it could represent a useful option in clinical practice since only a blood microvolume is required for the assessment (Schimojo *et al.*, 1993). The comparison between the analysis of pH and lactate in fetal scalp blood showed significantly fewer failures in sampling with lactate analysis and no differences in short-term neonatal outcome (Westgren *et al.*, 1998). Borruto *et al.* (2006) documented increased lactate levels in asphyctic infants and a clear correlation between lactic acidosis and fetal distress; furthermore, low Apgar scores were observed in neonates with moderate or severe asphyxia at birth. Concerning the influence of the type of delivery, lactate concentrations seemed higher in case of instrumental labor compared to spontaneous one. In distressed group, severe variable decelerations were generally recorded, by intra-partum cardiotocography, during the second stage of labor. The incidence of neonatal Apgar scores < or =7 in neonates with abnormal baseline fetal heart rate was higher than in those with severe variable decelerations, mild variable decelerations, and transient tachycardia. The duration of the active second stage of labor was significantly correlated with the presence of fetal lactate.

Also in canine species, the fetal viability evaluation is necessary above all towards the parturition date or when the bitch overcomes the predicted parturition day without signs of impending whelping (Veronesi, 2013). Newborn viability evaluation and early detection of fetal distress could contribute to reduce the mortality at birth, since in dogs a high neonatal mortality rate is reported subsequent to complicated and uncomplicated whelping (Groppetti *et al.*, 2010). Fetal heart rate is an excellent indicator of fetal stress. Normally, fetal heart rate is 2-3 times that of the bitch (220-240bpm) (Verstegen *et al.*, 1993; Zone and Wanke, 2001; Nyland and Mattoon, 2002). According to some authors, heart rate between 180 and 200 bpm is indicative of slight fetal stress (Zone and Wanke, 2001; Veronesi, 2013) and requires an obstetrical intervention within 2-3 hours, whereas rate consistently <180 bpm is typical of severe fetal distress

secondary to hypoxia (Zone and Wanke, 2001; Rickard, 2011; Veronesi, 2013) and the prognosis is usually inauspicious if there is no an immediate intervention (Veronesi, 2013). Nevertheless, other studies reported heart rates lower than the previous ones (<140-160 bpm) as markers of sustained fetal stress due to hypoxia (Verstegen *et al.*, 1993; Linde Forsberg, 2010); this cases require an immediate cesarean section (Linde Forsberg, 2010; Rickard, 2011). Intermittent uterine contractions can cause temporary, substantial reduction in fetal heart rate, but it should return normal within 1-2 minutes and remain within normal range if there is no fetal stress (Lopate, 2008). Zone and Wanke (2001) suggested the correlation between the fetal bowel movements and fetal compromise. In fact, bowel movements were observed in all the puppies with severe fetal distress, whereas in only 40% of puppies with slight fetal distress. According to some authors, the lack of fetal movements would be, independently by the fetal heart rate, a negative prognostic index for the canine species, as well as for humans (Veronesi, 2013). Fetal stress can also be detected by examining both the fetal fluids and feto-placental units (Zone and Wanke, 2001). At this regard, an increase in the echodensity of fetal fluids may indicate the premature placental separation with the consequent passage of meconium or hemorrhage into fetal fluids. Any variations in the fluids volume may suggest the rupture of fetal membranes, abnormalities of placental function or of fetal swallowing. Placenta edema or thickening may signal abnormalities or alterations in blood flow, decreased placental drainage, or placentitis (Lopate, 2008). IUGR may be suspected if abdominal:biparietal diameter ratio is less than 2 from day 48 to birth (Zone and Wanke, 2001).

According to Marchini *et al.* (2005), even the insulin-like growth factor binding protein-1 and interleukin-6 can be considered markers of fetal stress in human neonates during delivery at term gestation.

Finally, as in humans, umbilical vein lactate and tocodynamometry could provide valuable clinical information to improve the management of both mother and newborn, even in canine species. In both humans and dogs, fetal lactate level is considered an objective indicator of fetal distress and a valid predictor of neonatal survival. In fact, fetal acidosis recognition by the umbilical lactate measurement, combined to Apgar score classification and uterine activity monitoring during delivery, represents an advanced system in the evaluation of human and canine newborn patients. Umbilical lactate concentration was proved

to be useful to predict canine neonatal mortality within 48 hours from birth, using 5mmol/L of umbilical vein lactate as cut-off value to distinguish between healthy and distressed puppies. Higher values were related with distressed pups, whereas lower values characterized vigorous pups. Lactate concentrations lower than 5mmol/L and Apgar score higher than 9, related to mean delivery time of 105 minutes with effective uterine contractions (10mmHg of strength or more, frequency from 4 to 12 contractions per hour, and 2-5 min in duration) should be considered good prognostic factors in canine labor and neonatology (Groppetti *et al.*, 2010).

4.4 Neonatal adaptation

Despite several aspects of the neonatal physiology have to be still elucidated, the principle characteristics of canine neonatal physiology are already known. In mammals, the birth marks the fetal passage from the protective intrauterine life to the outside environment, characterized by multiple risk factors. The intrauterine environment guarantees, not only the exchanges by the placenta, but also the maintenance of a constant temperature and mechanical protection. During delivery, the fetus has to undergo several maturational and adaptation processes to ensure its independence from the mother. The feto-neonatal transition represents a critical phase, from which depends the following adaptation of the newborn to the extrauterine life. Any possible abnormality at delivery and, consequently during the neonatal phase, could lead to the survival failure in the first hours after birth or predispose the neonate to morbidity and mortality (Veronesi, 2013). Puppies are born much less mature than newborns of many other domestic species, and thus are more dependent on care during the first days of age. The neonatal physiology is characterized by an immature organ function, which undergoes dramatic changes, especially during the first 4-6 weeks after birth, to guarantee the correct adaptation to the extrauterine environment. A brief descriptions of the major changes occurring during the neonatal multi-systemic adaptation in the dog are therefore reported in the following paragraphs.

4.4.1 Cardiocirculatory system

The adaptation process of the cardiocirculatory system is probably the most clamorous event during the feto-neonatal transition. It is characterized by the breakdown of the placental circle, opening of the pulmonary circulation, and

closure of some typical fetal ways (Rickard, 2011). Several changes occur normally within few minutes or during the first hours/days after birth, leading to consequent blood flow variations, which are dramatically important to promote the pulmonary gas exchange, as well as to allow the neonatal survival.

4.4.2 Respiratory system

The key event for the transition to the extrauterine life is the beginning of the autonomous respiration. Fetal breathing movements, combined with the contractions of the intercostal muscles and diaphragm, occur already during the last phase of pregnancy to train the diaphragm in preparation for birth and promote the lungs growth. Furthermore, simultaneously to the adrenal gland functional development, the fetal cortisol secretion stimulates the final multi-organ maturation in preparation for postnatal life (Nathanielsz, 1998; Challis *et al.*, 2000; Veronesi, 2013). In the late 1960s, Liggins (1968-1969) was the first to suggest a positive effect of glucocorticoids on fetal maturation in lambs, reporting good results with the use of prenatal corticosteroids in mothers at risk of preterm delivery. Since then, many studies confirmed the clinical benefits of prenatal corticosteroids for prevention of respiratory distress syndrome. The exact mechanism of the lung maturation induced by glucocorticoid remains still unknown, but recent advances in molecular genetics increased the knowledge in human and lambs about the regulation of pulmonary development and surfactant production (McCormick and Mendelson, 1994; Ballard *et al.*, 1997; Reichardt *et al.*, 1998). In this respect, glucocorticoids probably act on structural lung growth and development (Schittny *et al.*, 1998; Whitsett and Stahlman, 1998), antioxidant enzymes (Saugstad, 1998), lung tissue growth factors (Saugstad, 1998; Jaskoll *et al.*, 1996), inflammatory mediators (Vyas and Kotheca, 1997), as well as on regulation of pulmonary absorption (Zhou *et al.*, 1996), beyond on stimulation of surfactant synthesis (Gross, 1990). Since the activation of the HPA axis is fundamental to maintain homeostasis in response to stress, a low secretory capacity of the adrenal cortex may cause a decreased stress response during acute illness in preterm newborns, with following increased morbidity (Bolt *et al.*, 2002; Watterberg, 2004). However, Fujitaka *et al.* (1997) suggested that premature infants are able to secrete glucocorticoids like at term newborns, even if in case of prematurity the fetal zone of the cortex, associated with a predominance of cortisone, remains functional for a longer time than in control. Scott and Watterberg (1995) found an inverse relationship between

gestational age and cortisol concentrations in preterm newborn babies, with the highest cortisol values in youngest infants and the lower values in subjects with the highest ventilatory requirements or that received surfactant, contrary to other authors which detected significantly increased basal cortisol concentrations in full-term infants with respiratory distress compared to normal newborns (Das *et al.*, 2002; Soliman *et al.*, 2004).

Since cortisol also works as the fetal trigger for the onset of calving, there is an efficient synchronization between the control of parturition and fetal lung maturation in cattle (Comline *et al.*, 1974; Hunter *et al.*, 1977). Because synthetic corticosteroids can pass the placental barrier, they can reach the fetal circulation when given to the mother, accelerating lung maturation in ovine (Houfflin-Debarge *et al.*, 2005) and surfactant production in calves (Zaremba *et al.*, 1997). Calves that are born before some 90% of the normal gestation length usually suffer from respiratory problems after birth, most likely associated with inadequate synthesis of surfactant (Bleul, 2009). Anyway, little is known about the physiological effects of prenatal glucocorticoid treatment on premature newborn calves. Normally cortisol concentrations, high at birth, tend to decrease during the first week of age and decline markedly after feed intake (Lee *et al.*, 1995; Hadorn *et al.*, 1997; Hammon and Blum, 1998). Neonatal calves born by cesarean section seem to be more predisposed to develop a respiratory distress syndrome, and therefore a respiratory acidosis, during the first hours of life (Cambier *et al.*, 2000). Some authors found higher cortisol levels in fetal lambs affected by hypoxemia compared to controls (Gardner *et al.*, 2001; Gardner *et al.*, 2002). In foal and lambs, since fetal cortisol increases late in gestation, cardiovascular and endocrine function maturation could be delayed, and the cortisol increases during the early postnatal life to continue these maturational events (O'Connor *et al.*, 2005). Panzani *et al.* (2009) reported high cortisol levels in foals at birth and a decline to basal values within 3 hours after foaling, in agreement with other studies (Rossdale *et al.*, 1982; Silver *et al.*, 1991), in which premature foals exhibited significantly low cortisol concentrations. Moreover, no postnatal rise in cortisol levels was observed in these subjects.

4.4.3 Digestive system

At birth, the digestive system of the newborn is adequate only for the digestion and absorption of the maternal milk, with an increased enzymatic activity just

before parturition to ensure a full efficiency in the first hours of life, and undergoes changes in parallel to the variations of maternal secretions. However, the neonatal digestive system is immature both structurally and functionally. In newborn puppy the muscular layer of the small intestine is about 50% thinner compared to that found in adult dog. Some pancreatic enzymes are lacking, as well as the α-glycosidases are not well developed, with subsequent problems for the digestion of sucrose and maltose. The digestive capacity of the stomach and pancreas increases gradually paralleling to the puppies age (Casal, 2010; Peterson, 2011; Rickard, 2011; Veronesi, 2013).

After birth, the gastrointestinal system is steril; in the first 24 hours of age, it undergoes a bacterial colonization, which gives rise to the microbial flora with progressive increase of anaerobic bacteria (Casal, 2010; Fitzgerald and Newquist, 2011; Rickard, 2011; Veronesi, 2013). The intestinal permeability to macromolecules declines about 24-48 hours after birth, likely due to the rapid maturation of the intestinal wall, which acquires hydrolytic properties against macromolecules and becomes a protective barrier against the "bacterial translocation". Since the third week of age, some important changes of the digestive system occur in association with the replacement of milk by solid food, such as the doubling of the intestinal wall thickness. The introduction of solid food provides both a source and substrate for new bacterial growth, which replace those induced by milk feeding and constitute the final intestinal microbial flora (Peterson, 2011; Veronesi, 2013).

At birth, also the liver is not able to perform neither gluconeogenesis nor glycogenolisis, and the mechanisms of biotransformation, detoxification, and elimination are reduced (Veronesi, 2013). Many of the microsomal enzymes, generally involved in drug metabolism, are not functional during the neonatal period. In fact, the liver acquires the almost complete functional capacity from the 8 weeks of age onward (Casal, 2010; Fitzgerald and Newquist, 2011; Root Kustritz, 2011).

4.4.4 Urinary system

At birth, the nephrogenesis is not complete but continues until 3 weeks of age, with structural and functional changes in all kidney components (Casal, 2010; Fitzgerald and Newquist, 2011; Root Kustritz, 2011).

4.4.5 Muscular and skeletal system

Most of the maturation and growth processes going on in the newborn already start during intrauterine life, closely regulated by maternal and fetal endocrine mechanisms. Along human pregnancy, the placenta appears strictly involved in the regulation of fetal growth (Murphy *et al.*, 2006). There are little data to suggest a direct role for estrogen and progesterone in fetal growth regulation, but some studies demonstrated some correlations between the concentrations of these hormones and birth weight or placental weight (Mucci *et al.*, 2003; Mucci *et al.*, 2004).

After birth, the newborns show a creeping movement because of the neurologic, skeletal, and muscular immaturity. Regarding the skeleton, "fontanellae" are detectable on the skull. Along the neonatal and pediatric periods, there is a progressive growth of cartilagineous elements and bone mineralization, with remodeling influenced by simultaneous neurologic development (Veronesi, 2013). Normal puppies can lift their head already at birth, try to push themselves up on their forelimbs or scoot along using their hindlimbs by about 10 days of age, and attempt to walking by 14 days (Root Kustritz, 2011). However, a real locomotory capacity is reached by the third week of age.

4.4.6 Immune system

After birth, the exposure to the new environment and foreign antigens requires the establishment of appropriate defense responses. The neonatal immunity competence is not so much efficient until 6 weeks of age, and it becomes fully efficient at about sixteen weeks. In the bitch, the endotheliochorial placenta allows a limited transfer of immunoglobulins to fetus; in fact, in the dog only 5-10% of the neonatal antibodies derives from the transplacental passage during the last third of pregnancy. Consequently, the newborn puppies are dependent on the passive immunity derived from the colostrum, produced in the first 36-48 hours after whelping (Chappuis, 1998; Day, 2007; Casal, 2010; Evermann and Wills, 2011; Fitzgerald and Newquist, 2011; Rickard, 2011; Veronesi, 2013). The colostrum provides several nutritional substances, included vitamins and electrolytes, and allows the transfer of passive immunity, such as immunoglobulins, mainly IgG and IgA, and less IgM, and bioactive substances, such as the lysozyme, lattoferrin, lattoperoxidase, interferon, and trypsin inhibitor, oligosaccharides complexes, mucines, cytokines, chemokines, and

leucocytes (Fitzgerald and Newquist, 2011; Veronesi, 2013) . Furthermore, the colostrum contains the lipase, which promotes the lipids digestion. The particular structural and functional characteristics of the digestive system ensure, immediately after birth, the intestinal permeability to these macromolecules, allowing the transfer of the passive immunity. Although not specifically documented in the dog, it is assumed that this transfer takes place thanks to different mechanisms, such as low concentration of intestinal proteolytic enzymes and the transient expression of the intestinal FcγR, immunoglobulin receptor, allowing absorption of IgG into the neonatal vascular and lymphatic circulations (Day, 2007). IgM and IgA are also absorbed, but it is not specifically known whether colostrum IgA are significantly absorbed and re-secreted or largely remain within the intestinal lumen in this species (Day, 2007). In canine species, gut "closure" seems to begin as early as 4-8 hours after birth and to be complete within 16-36 hours (Casal, 2010; Evermann and Wills, 2011; Fitzgerald and Newquist, 2011; Rickard, 2011; Chastant-Maillard *et al.*, 2012). This could be the result of changes in intestinal pH and proteolytic enzymes, as well as the loss of specific receptors (Casal, 2010). The immunoglobulins concentration is maximal at delivery, but declines drastically in the following 2 days, paralleling to the transition from colostrum to milk (Evermann and Wills, 2011; Veronesi, 2013). The maternally derived antibodies may last until 6-16 weeks after birth, then they decrease to undetectable limits (Evermann and Wills, 2011). As maternal antibody decreases, endogenous immunoglobulins secretion steadily increases. It is well known that, unfortunately, the presence of maternal antibodies represents a clear obstacle to the effectiveness of vaccines in the neonatal period; in fact, the "blanketing" phenomenon refers to the neutralization of the vaccine antigen by the maternal antibodies. Furthermore, in vaccinated puppies the maternal immunity inhibits the immune response against the vaccine antigens for which the passive antibodies are specific. As maternal antibodies are decreasing, there is a "susceptibility window" in which maternal immunoglobulins are not enough to ensure an adequate immunity protection, but are enough to interfere with the active immune response in the newborn at vaccination (Chappuis, 1998; Day, 2007; Morein *et al.*, 2007; Evermann and Wills, 2011). Unfortunately, the beginning and duration of this critical period are very changeable among different litters and even among the puppies within the same litter (Veronesi, 2013). The point at which a newborn puppy becomes immune competent (generally between 6 and 12 weeks of age) is thus

determined by the concentration of colostrum immunoglobulin ingested. Puppies not receiving colostrum are able to mounting a protective immune response much sooner compared to those with maternal passive immunity, as early as 2 weeks of age (Day, 2007). In general, puppies begin life with a competent but immature immune system that matures over the first 6 to 12 months of life (Chappuis, 1998; Evermann and Wills, 2011), but the development of the immune system remains still to clarify in canine species, since the substantial changes are poorly documented (Day, 2007).

4.4.7 Neonatal mortality

The neonatal adaptation represents one of the most critical phase during the entire life of a living being, so that any failure along this process or some peculiar features of the same newborn can potentially cause the death of the subject. The reported neonatal mortality rate in dogs ranges from 9 to 34% subsequent to complicated and uncomplicated whelping, with a greatest risk during the first week of age (Davidson, 2003; Peterson, 2011). Neonatal death was documented to be firstly due to dystocia (Münnich *et al.*, 1996; Moon *et al.*, 2000; Moon-Massat and Erb, 2002), whereas the bacterial infection was recognized to be the second main cause of puppies mortality, since it frequently evolves to septicaemia within the first 14-21 days of age (Sager and Remmers, 1990; Poffenbargerm *et al.*, 1991; Münnich *et al.*, 1995; Van der Beek *et al.*, 1999; Davidson, 2003; Daniels and Spencer, 2011). Several factors should be considered in the predisposition to neonatal septicaemia, among which the bitch, whelping, environment, the same newborn, as well as the other subjects living in the breeding (Sager and Remmers, 1990; Poffenbargerm *et al.*, 1991; Go *et al.*, 1994; Münnich *et al.*, 1995; Van der Beek *et al.*, 1999; Johnston *et al.*, 2001; Davidson, 2003; Peterson *et al.*, 2011). The infection often spreads from mother to fetus during gestation, delivery or, after whelping, through infected maternal secretions, such as vaginal and oronasal discharges, faeces, and milk (Sager and Remmers, 1990; Münnich, 2004). Bacterial translocation, that is the passage of live bacteria through intact intestinal barrier, is recognized as another cause of systemic disease, above all in newborn (Go *et al.*, 1994; Dahlinger *et al.*, 1997).

In canine species, the microbial organisms most frequently associated with neonatal mortality are *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, *Streptococcus canis*, *Streptococcus dysgalactiae* subsp *equisimilis*, and

Streptococcus equi subsp *zooepidemicus*, and *Klebsiella pneumoniae* (Askaa *et al.*, 1978; Mosier, 1981; Sager and Remmers, 1990; Johnston *et al.*, 2001; Davidson, 2003; Daniels and Spencer, 2011).

The neonatal septicaemia can have a hyperacute or subacute evolution. The hyperacute form is often characterized by the asymptomatic death of the newborn puppy (Askaa *et al.*, 1978; Davidson, 2003; Daniels and Spencer, 2011), whereas in case of subacute course the newborn, apparently healthy and with a normal weight at birth, dies within the first days of age, showing really unspecific symptoms (Johnston *et al.*, 2001). Typical clinical signs include decrease in weight gain, loss of the sucking reflex, weakness, cyanosis, persistent watery diarrhea, hematuria, unusual vocalizations, abdominal distension and/or pain, hypothermia, sloughing of the extremities, and coma (Johnston *et al.*, 2001; Davidson, 2003). The clinical management of infected newborn puppy is very difficult because of the fast onset of unspecific symptoms and disease course; thus, prognosis is often poor (Johnston *et al.*, 2001; Davidson, 2003).

CHAPTER 5

Non-invasive methods for perinatal investigations

5.Non-invasive methods for perinatal investigations

Because the recent increasing interest in small animals neonatology, the development of specific methods for assessing both fetal and neonatal well-being became necessary to ensure an early diagnosis of abnormal pregnancy or fetal distress or to improve the management of pathologic newborns, respectively. At this regard, as in the other domestic species, the non-invasive techniques would be more advisable in the respect of the animal welfare.

Since the last decades, the early stages of gestation were monitored in bovine species by ultrasonography (Curran *et al.*, 1986; Muller and Wittkowski, 1986), contrary to the final phases of pregnancy, during which the uterus and fetus were not so widely assessed. Along the last trimester of gestation, the indirect evaluation of the bovine conceptus is possible through the fetal fluids and placental membranes observation (Buczinski *et al.*, 2007; Baska-Vincze *et al.*, 2014). Despite placental investigation and placentomes size detection by ultrasounds could be useful in assessing any abnormality in fetal growth during late pregnancy in cattle (Wallace *et al.*, 2000), to the author knowledge these methods were not commonly employed (Heyman *et al.*, 2002), in contrast to mares (Reef *et al.*, 1996) and ewes (Anthony *et al.*, 2003) which worked as animal models for human research. In both humans (Manning, 1999) and horses (Reef *et al.*, 1995; Reef *et al.*, 1996) ultrasonographic biophysical profiles were reported and are currently used in practice. Particularly, anomalies of the utero-placental unit were demonstrated to be important factors in the detection of fetal well-being in diseased mares (Reef *et al.*, 1996; Pantaleon *et al.*, 2003).

Beyond the placenta, also abnormalities in fetal fluids can reflect pathologic conditions, and transabdominal ultrasonography could be useful as a diagnostic tool in several species (Buczinski *et al.*, 2007; Baska-Vincze *et al.*, 2014). Hydrallantois is a common hydropic condition in cattle and it is due to placental anomalies, whereas hydramnios is usually secondary to congenital fetal abnormalities (Troy, 1993). Both these conditions are detectable by ultrasounds, even if a precise description was not yet documented (Buczinski *et al.*, 2007). Even a decreased fetal fluids amount is an abnormal finding in humans and horses; in fact, human oligohydroamnios is frequently associated with intrauterine growth retardation, intra-partum asphyxia, and fetal death (Moore, 1997), whereas in equine species a decrease in fetal fluid depth often indicates a

poor foal outcome (Reef *et al.*, 1996). Additionally, in mares, abnormal echogenicity of amniotic fluid was documented in case of placentitis, septicaemia, and peri-partum asphyxia syndrome (Reef *et al.*, 1996), as well as in ruminants, in which the repeated observation of debris in fetal fluids may mean a compromised fetus (Jonker, 2004). Finally, more recently the equine amniotic fluid and blood were collected to evaluate the lactate concentrations in both mares and foals in early post-partum period, since the lactate was judged as an important marker of hypoxia and a useful indicator for prognosis in horse foals (Pirrone *et al.*, 2012).

In canine species, fetal fluids ultrasonographic characteristics along normal or pathologic pregnancies were scarcely investigated. To the author knowledge, only Zone and Wanke (2001) found a significant correlation between the fetal compromise and both the fetal fluids and feto-placental units. Interestingly, an increase in the fetal fluids echodensity may indicate the premature placental separation with following passage of meconium or hemorrhage into fetal fluids, whereas any variations in the fluids amount may suggest the rupture of fetal membranes, anomalies of placental function or of fetal swallowing. Placenta edema or thickening may signal abnormalities or alterations in blood flow, decreased placental drainage, or placentitis. Furthermore, the intrauterine growth retardation may be suspected if the abdominal:biparietal diameter ratio is less than 2 from day 48 to birth (Zone and Wanke, 2001). Also the fetal fluids composition in dogs received scarce scientific interest. Likely to the horse, more recently the umbilical vein lactate level was proved to be an objective indicator of fetal distress and a valid predictor of neonatal survival even in dogs (Groppetti *et al.*, 2010).

Among the several important functions, another key role of the amniotic fluid was described in cattle and humans regarding the estimation of fetal lung maturity, based on the lecithin to sphingomyelin ratio (Eigemann *et al.*, 1984; Moore, 1997) or the lamellar body count, that still remains valuable in human neonatology (Roiz-Hernandez *et al.*, 2002). The amniotic fluid collection is performed by amniocentesis, which is considered safe in women (Norwitz and Levy, 2013) contrary to cows and mares (Schmidt *et al.*, 1991; Callan *et al.*, 2002). Despite in women both amniocentesis and chorionic villus sampling are commonly performed, non-invasive testing are developing to avoid direct contact with the growing fetus/placenta and all the related risks. Among these,

the first-trimester risk assessment, maternal serum analyte screening, and sonographic fetal structure survey are included to verify the risk of a fetal aneuploidy. The first-trimester risk assessment is generally performed at 11-14 weeks pregnancy through the sonographic nuchal translucency and the measurement in maternal serum of β-human chorionic gonadotropin (β-HCG) and pregnancy-associated plasma protein-A, whereas the maternal serum analyte screening is usually offered at 15-20 weeks gestation and it measures the circulating levels of alpha-fetoprotein, β-HCG, unconjugated estriol, and inhibin-A in maternal serum. Finally, the sonographic fetal structure survey, commonly offered at 18-20 weeks pregnancy, allows to identify possible major structural defects, often associated to chromosomal abnormality, or typical soft markers of fetal aneuploidy (Norwitz and Levy, 2013).

Beyond the fetal annexes, also the assessment of the fetus at term represents a reliable method to detect some anomalies. In humans, the fetal breathing and body movements, as well as the fetal heart rate and heart rate variability, seem to be suitable as good markers of fetal well-being (Bocking, 2003), whereas in equine species, several parameters, such as the fetal heart rate, fetal breathing and body movements, fetal aortic diameter, and fetal eye size were investigated by non-invasive techniques (fetal electrocardiography, transabdominal Doppler and ultrasonography) (Reef *et al.*, 1996; Pantaleon, 2003). The fetal breathing and body movements were documented even in ewes (Bocking and Harding, 1986), whereas only limited data are available about these parameters in cattle (Bocking, 2003). In canine species both the correlation between the fetal heart rate and fetal compromise (Verstegen *et al.*, 1993; Zone and Wanke, 2001; Nyland and Mattoon, 2002), and between the fetal bowel movements and fetal distress (Zone and Wanke, 2001), were studied. However to date, the fetal heart rate appears the safest parameter to assess the fetal well-being towards term of gestation in several species, even if its interpretation is often complicated, as in humans (Manning, 1999), horse (Reef *et al.*, 1996; Nagel *et al.*, 2010; Baska-Vincze *et al.*, 2015), sheep (George *et al.*, 2004), cattle (Jonker *et al.*, 1993; Jonker *et al.*, 1996; Baska-Vincze *et al.*, 2014), and dog (Verstegen *et al.*, 1993; Zone and Wanke, 2001; Nyland and Mattoon, 2002). Nevertheless, in human beings, recently fetal scalp blood lactate was recognized as the best predictor of severe neonatal morbidity and intra-partum surveillance, since it represents the perfect

marker of hypoxia (Kruger *et al.*, 1999; Wiberg-Itzel *et al.*, 2008; East *et al.*, 2010; Wiberg *et al.*, 2010; Holzmann *et al.*, 2011).

Although the majority of the above mentioned tools are related to fetal normal growth, it should be stressed that the term "growth" strongly differs from the concept of "maturity". Determining if the newborn is sufficiently mature for the extrauterine life is of critical importance for its outcome. In this respect, investigations performed on non-invasive biological matrices seem a promising technique to assess fetal and neonatal adaptation and well-being (Kindahl *et al.*, 2002; Buczinski *et al.*, 2007).

CHAPTER 6

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CHAPTER 7

Objectives

7. Objectives

In human medicine, beyond the ultrasounds examination, the monitoring of the fetal fluids characteristics represents the best tool to investigate the fetal and neonatal well-being. To the author knowledge, this interesting topic was more or less deeply documented in all domestic animals, except in canine species. Particularly, the characteristics of the fetal fluids during the different stages of the normal pregnancy, as well as the possible fetal fluids changes correlated to fetal diseases, are totally lacking in dog. The present thesis represents only a starting point, aimed to investigate the features of the fetal fluids belonging to normal, viable, and well developed newborn puppies born by normal term gestations. Specifically, the insulin-like growth factors-I and non-esterified fatty acids concentrations were assessed in amniotic and allantoic fluids because of their critical role in fetal and neonatal well-being and development. Furthermore, as their protective fetal role, also the immunoglobulin G and lysozyme levels were measured in both fetal fluids. Further researches are needed to verify any possible correlation between the fetal fluids composition and fetal development anomalies or maternal and fetal/neonatal diseases in general, and to find any possible relationship between the specific maternal or fetal pathological conditions and the presence, as well as the amount, of some substances. Additionally, it would be interesting to investigate the possible relationship between the fetal fluids anomalies and neonatal impaired viability.

Since the not invasive techniques are considered more advisable to investigate the fetal and neonatal well-being, beyond the fetal fluids, the hair and nails appear as the newest biological matrices in which hormonal concentrations measurement is possible. Hair and nails cortisol levels represent the perfect biomarker for monitoring the HPA axis activity over long periods. In fact, along their growth, both hair and nails accumulate this hormone from blood, thus providing a retrospective picture of previous long-term cortisol accumulation. To date, little is known about hair cortisol analysis in canine species, whereas the nails cortisol concentrations were never assessed. Also in this case, the present thesis represents only a starting point, aimed firstly to verify the hormone levels detectability in both hair and nails belonging to normal developed dead newborn puppies. Further researches are obviously needed to better investigate the cortisol concentrations in case of prematurity or fetal diseases, as well as to study

hormonal variations in both healthy and sick puppies during the neonatal and pediatric periods. Moreover, it could be interesting to perform the same analysis even on the dam hair to clarify the real origin of cortisol and the effects of maternal conditions on newborns hair hormone levels.

It is well known that the neonatal period in dog represents a phase of high susceptibility to bacterial infection, since the immune competence of puppies is not fully achieved until several weeks after birth. Indeed, the septicaemia was identified as the main cause of neonatal death during the first 2-3 weeks of age and it can show a hyperacute or subacute course. In both cases, the medical management of the infected newborn puppy is really difficult because of the sudden onset of unspecific clinical signs and the fast fatal disease course. Despite the high percentage of neonatal losses in canine species, in the last years only few studies investigated the role of bacterial infections in newborn puppies mortality. For this reason, the present thesis aimed to clarify the real involvement of bacterial infections in neonatal mortality in dog, beyond to evaluate the antibiotics susceptibility of the isolated bacteria to improve the clinical management of both litter and dam in case of neonatal septicaemia.

In canine species, also the information available about the skeletal development are limited, above all along the first month of age, and the previous studies about the evaluation of the ossification centers appearance and fusion are difficult to compare each other because, in most cases, the breeds enrolled, age and method of examination, anatomical compartments investigated, as well as the aim of the researches, do not appear homogeneus. Thus, the present research was aimed to study the normal neonatal growth and to find new, easily performable methods to estimate the biological age of the puppies, often illegally imported younger than 2 months of age. At this regard, it could be useful the creation of a radiographic data-base concerning the ossification centers appearance during the first month of age in puppies. Indeed, the last aim of the present thesis was to verify the timing appearance of hindlimb ossification centers, as well as the radiographic and anatomical morphometry of the hindlimb long bones, skull, and body, in large-giant sized puppies dead spontaneously within the first 30 days of age.

General aims

1. IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs.
2. Immunoglobulin G and lysozyme concentrations in fetal fluids of term pregnancy dogs.
3. Hair and nails as new, non-invasive matrices for long time-frame cortisol analysis in newborn dogs.
4. A survey on bacterial involvement in neonatal mortality in dogs.
5. Age estimation in large and giant newborn puppies through the hindlimb ossification centers evaluation and morphometry of hindlimb long bones, skull, and body.

CHAPTER 8

Insulin-like growth factor-I
and
non-esterified fatty acids

8.1 Introduction

Towards the end of pregnancy and during the early postnatal period, the newborn puppies begin the adaptation to the extrauterine life, going to meet several physiological changes. This process occurs despite incomplete maturation of their endocrine system, which continues its development along the postnatal phase. Under the regulation of HPA axis, several hormones and growth factors work together to ensure the normal somatic growth.

In mammals, fetal growth and well-being are regulated by many factors and the placenta contributes to maintain the best environment for the fetus. The fetal fluids, especially the amniotic fluid, should be considered as a marvelously complex and dynamic milieu that changes along pregnancy; in fact, it is recognized to contain important factors involved in fetal growth and development, and even in fetal protection (Underwood *et al.*, 2005).

8.2 Insulin-like growth factor-I

Among these substances, growth factors, such as IGF family, were widely studied in the last decades. In mammals, the insulin-like growth factors (IGF-I and IGF-II) hold a crucial role in promoting fetal and postnatal growth (Monzavi and Cohen, 2002; Randhawa and Cohen, 2005), brain and inner ear development (Netchine *et al.*, 2011), and metabolism (Randhawa and Cohen, 2005). In this respect, previous studies revealed that mice with a homozygous defect of IGF-I gene showed a severe embryonic and postnatal growth retardation (Baker *et al.*, 1993; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993), beyond neurological defects (Beck *et al.*, 1995). Furthermore, cases of humans with complete IGF-I deficiency, due to a gene deletion, were characterized by low weight at birth, growth failure, mental retardation, and sensorineural deafness (Woods *et al.*, 1996; Camacho-Hubner *et al.*, 2002). When fetal growth is concerned, IGF-I seems to play a more important role than GH (Collett-Solberg and Cohen, 2000; Cohen and Rosenfeld, 2002), such as documented by the mating between GH-deficient mice and IGF-I transgenic mice that gave rise to offspring with normal body weight and linear growth (Behringer *et al.*, 1990). Moreover, IGF-I has an essential role in post-natal growth, as supported by a severe post-natal growth arrest in mice with IGF-I gene deletion (Baker *et al.*, 1993; Liu *et al.*, 1993).

In human beings, the intrauterine secretion of IGFs occurs primarily in response to fetal nutrition and insulin levels (Chard, 1994), and the IGF-I concentrations are strongly regulated within each tissue, based on its developmental stage (Allan *et al.*, 2001; Monzavi and Cohen, 2002). In childhood and adult life IGF-I is secreted by the liver, under GH control and on the basis of nutritional status (Netchine *et al.*, 2011), whereas IGF-II release is GH-independent (Monzavi and Cohen, 2002). IGF-I production increases during the last trimester of pregnancy in humans (Gluckman *et al.*, 1983; Lassarre *et al.*, 1991; Monzavi and Cohen, 2002) and sheep (Gluckman and Butler, 1983), and continues after birth (Gluckman *et al.*, 1983; Gluckman and Butler, 1983; Randhawa and Cohen, 2005; Netchine *et al.*, 2011), with a peak at puberty. Differently, IGF-II concentrations are low at birth, increase in the first week of age, and then remain stable (Monzavi and Cohen, 2002). In humans, IGFs activity is regulated by IGF binding proteins (IGFBPs) and IGFBP proteasis (Monzavi and Cohen, 2002), but unfortunately how these hormones regulate the fetal growth remains poorly understood (Randhawa and Cohen, 2005).

IGFs seem to be involved in common pathological processes, such as fetal growth restriction (FGR) in humans and mice, due to mutations or targeted deletions of the IGF ligands, as well as the IGF-I receptor and its main signaling molecule (Randhawa and Cohen, 2005). Specifically, it was suggested that homozygous defect of IGF-I gene in mice (Baker *et al.*, 1993; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993) and IGF-I gene deletion in humans (Woods *et al.*, 1996; Camacho-Hubner *et al.*, 2002) are responsible for this fetal pathology. In the early neonatal period, umbilical cord serum (Lassarre *et al.*, 1991; Verhaeghe *et al.*, 1993; Giudice *et al.*, 1995; Ostlund *et al.*, 1997), serum (Leger *et al.*, 1996), or plasma (Ohkawa *et al.*, 2009) IGF-I concentrations were closely correlated with gestational age at birth (Verhaeghe *et al.*, 1999), birth weight and size (Verhaeghe *et al.*, 1993; Ostlund *et al.*, 1997; Ohkawa *et al.*, 2009), and pathologic conditions, such as intrauterine growth retardation (IUGR) (Lassarre *et al.*, 1991; Giudice *et al.*, 1995; Leger *et al.*, 1996; Ostlund *et al.*, 1997). Interestingly, after birth, low IGF-I levels were found in IUGR and in small-for-gestational-age (SGA) babies (Lassarre *et al.*, 1991; Verhaeghe *et al.*, 1993; Giudice *et al.*, 1995; Leger *et al.*, 1996; Ostlund *et al.*, 1997; Ogilvy-Stuart *et al.*, 1998; Monzavi and Cohen, 2002; Ohkawa *et al.*, 2009), whereas high IGF-I concentrations were detected in large-for-gestational-age (LGA) infants (Lassarre *et al.*, 1991; Verhaeghe *et al.*, 1993;

Giudice *et al.*, 1995; Monzavi and Cohen, 2002). IGF-I null mice showed retarded growth and defects in development of brain, muscle, bone, and lung, and were sterile, whereas in humans the genetic IGF-I defects caused prenatal and postnatal growth retardation, sensorineural deafness, and intellectual deficit (Netchine *et al.*, 2011). In humans and sheep, a negative correlation was reported between IUGR and IGF-I in amniotic fluid (Gomez Roig *et al.*, 2005; Eremia *et al.*, 2006). Additionally, in women a difference in amniotic fluid IGF-I concentrations was documented between normally developed and IUGR pregnancies, and those levels were thus related to birth weight (Delmis *et al.*, 1992). IGF-I plasma concentrations were measured also in term foals and no differences were found on the basis of birth weight or gender (Panzani *et al.*, 2013), as opposed to the human babies in which females showed higher circulating IGF-I levels than males (Ibáñez *et al.*, 2008). Moreover, premature foals did not have lower IGF-I concentrations when compared to healthy term ones (Panzani *et al.*, 2013), whereas several studies performed on human neonates documented that prematurity was characterized by lower IGF-I plasma levels (Giudice *et al.*, 1995). Even in sheep fetus, IUGR was associated with decreased fetal IGF-I concentrations (Oliver *et al.*, 1996).

The literature reports that IGFBP-3 represents the primary binding protein, extending the IGF half life in circulation (Randhawa and Cohen, 2005), whereas elevated IGFBP-1 levels inhibite fetal growth, probably by sequestering fetus-derived IGF-I (Chard, 1994). At this purpose, several studies revealed that decreased concentrations of IGF-I (Giudice *et al.*, 1995; Leger *et al.*, 1996), IGFBP-3 (Giudice *et al.*, 1995; Leger *et al.*, 1996), with increased levels of IGFBP-1 (Verhaege *et al.*, 1993; Chard, 1994; Ostlund *et al.*, 1997), were associated with IUGR. Especially, IGFBP-1 concentrations in fetal cord serum collected in the third trimester appeared higher in term SGA babies and in preterm AGA newborns than in term AGA ones (Verhaeghe *et al.*, 1993; Ostlund *et al.*, 1997; Verhaeghe *et al.*, 1999), whereas IGFBP-3 levels seemed to be lower in IUGR neonates (Giudice *et al.*, 1995). On the contrary, LGA infants showed elevated IGF-I and IGFBP-3, and decreased IGFBP-1 (Giudice *et al.*, 1995). Even in placenta, decreased expression levels of hPGH, IGF-I, and IGFBP-1 were found in case of pregnancies complicated by FGR (Koutsaki *et al.*, 2011).

8.3 Non-esterified fatty acids

Fetal growth and development require an adequate supply also of fatty acids, that represent important constituents of the membrane lipids and essential intracellular mediators of gene expression (Jump, 2004; Sampath and Ntambi, 2004; Hulbert *et al.*, 2005; Schmitz and Ecker, 2008).

The fetus depends on the maternal supply of long-chain polyunsaturated fatty acids (LCPUFA) for its growth and development. During late pregnancy, the enhanced lipid catabolism causes a maternal hyperlipidemia, giving LCPUFA available for the fetus (Duttaroy, 2009). Little is known about the transfer of these molecules from placenta to fetal circulation (Gil-Sánchez *et al.*, 2011), but the placental lipoprotein lipase (LPL) activity seems to be essential for the provision of free fatty acids to the fetus; in fact, placental LPL function appears reduced in preterm IUGR (Magnusson *et al.*, 2004). The fatty acids deficiency during embryological organogenesis may be devastating, above all for neurological development (Duttaroy, 2009). Previous studies investigated the relationship between maternal docosahexaenoic acid intake along gestation and lactation and cognitive functions in later childhood (Helland *et al.*, 2003; Jensen *et al.*, 2005), demonstrating that children of women with low fish intake during pregnancy had an increased risk of poor cognitive and behavioural outcome (Oken *et al.*, 2005; Hibbeln *et al.*, 2007). In humans it was documented that, at birth, the non-esterified fatty acids (NEFA) concentrations appeared higher in umbilical vein compared to umbilical artery, and positively correlated to maternal levels (Lewis *et al.*, 2011). Concentrations of NEFA were assessed also in healthy and pathologic newborn foals, born by spontaneous or induced parturition, to evaluate how the type of delivery and health status could influence the metabolic profile (Panzani *et al.*, 2012). Regarding the fatty acids level in human amniotic fluid, a previous study reported that no correlation existed between amniotic fluid free fatty acids (FFA) and maternal plasma ones, suggesting that amniotic fluid FFA may partially derive from fetus renal excretion (Hagenfeldt and Hagenfeldt, 1976). Furthermore, it was demonstrated that, in human pregnancy complicated by IUGR, FFA amount in amniotic fluid is almost three times higher than in normal gestation (Urban and Iwaszkiewicz-Pawlowska, 1986).

Fetal fluids amount and composition were largely studied in both humans and some domestic animals, but few data are available about their composition in

canine species. In particular, to the author knowledge, IGF-I and NEFA were never investigated in dog fetal fluids. For these reasons, the present study was aimed to verify the presence of IGF-I and NEFA in both amniotic and allantoic fluids, collected from normal developed and viable newborn puppies born at term of normal pregnancies, evaluating possible differences in their concentrations between the two fluids and finding any possible correlation between their levels and breed size or fetal gender.

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CHAPTER 9

IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs

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9. IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs

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9.1 Abstract

Insulin-like growth factor-I (IGF-I) and non-esterified fatty acids (NEFA) play an essential role in fetal growth and development. To date, fetal fluids IGF-I and NEFA levels at term canine pregnancy are unknown and could be related to the neonatal development and breed size. For these reasons, the aims of the present study were as follows: 1) to evaluate IGF-I and NEFA concentrations in fetal fluids collected from normally developed and viable newborn puppies born at term of normal pregnancies; 2) to assess possible differences between IGF-I and NEFA levels in amniotic compared to allantoic fluid; 3) to detect possible relationship between breed body size and IGF-I and NEFA amniotic and allantoic concentrations; 4) to evaluate possible differences in IGF-I fetal fluids levels between male and female puppies; and 5) to assess possible correlations between the two hormones in each type of fluid. The study enrolled 25 pure breed bitches submitted to elective Cesarean section at term because of the high

risk of dystocia or previous troubles at parturition. At surgery, amniotic and allantoic fluids were collected and assayed for IGF-I and NEFA. IGF-I and NEFA amounts in both amniotic and allantoic fluids of different breed size bitches (small: ≤10 kg; medium: 11-25 kg; large: 26-40 kg) were detected, as well as the effect of gender on IGF-I levels. On a total of 73 amniotic and 76 allantoic samples collected by normal, viable, and mature newborns, the mean IGF-I concentration was significantly higher in amniotic than in allantoic fluid in all three groups, but the amniotic IGF-I levels were significantly lower in small and medium size bitches when compared with large ones. No significant differences were found in allantoic IGF-I concentrations among size groups. A significant effect of the puppy gender on IGF-I content in both fetal fluids was not reported. Regarding NEFA, in all the three groups, the mean NEFA concentration did not significantly differ between amnion and allantois, but in both fetal fluids, higher NEFA levels were detected in samples belonging to small breeds when compared with medium and large. These data strongly indicated that, also in the dog, a relation between fetal fluids IGF-I and NEFA concentrations and breed size exists. Further research is needed to elucidate the possible role of IGF-I and NEFA in the pathologic conditions related to canine fetal growth.

9.2 Introduction

In the last years, an increasing interest for the canine neonatology, mainly aimed to the improvement of after birth survival and to the correct management of the newborn puppies, was observed. In mammals, the newborn is the result of a prenatal intrauterine development, which in the last phase of gestation involves a lot of complex mechanisms leading the fetus to be ready for the challenging neonatal adaptation [1-3].

In contrast to humans [4-6], literature about canine fetal fluids composition is scarce and the role of amniotic compounds involved in the process of fetal growth and development, as well as in the fetal well-being and in preparation for birth, was not yet investigated. Therefore, amniocentesis is still an unused procedure for the routine assessment of fetal well-being or for prenatal diagnosis of diseases in dogs.

In the last decades, among the multitude of substances detectable in fetal fluids, growth factors, such as IGF family, were examined in animal models, focusing

on human neonatal studies. In mammals, in fact, the insulin-like growth factors (IGF-I and IGF-II) have a crucial role in promoting fetal and postnatal growth [7, 8], brain [9-12] and inner ear development [9], and metabolism [7]. Insulin-like growth factors-I is also supposed to be involved in fetal diseases, such as the intrauterine growth retardation (IUGR), in humans and mice [7]. In the early human neonatal period, umbilical cord serum [13-16], serum [17], or plasma [18] IGF-I levels were closely related with birth weight and size [13, 15, 18], and eventual IUGR [13, 14, 16, 17]. Additionally, higher amniotic fluid IGF-I concentrations were demonstrated in normally developed infants when compared with IUGR neonates and it was, therefore, supposed that IGF-I could be related to birth weight [19]. Plasma IGF-I levels were measured also in term foals and no difference were noted on the basis of birth weight or gender [20], in contrast to the human babies, in which females showed higher circulating IGF-I concentrations than males [21]. In the sheep, IUGR fetuses were found to be associated with decreased circulating IGF-I levels [22].

In humans, the fetus totally depends on the maternal supply of long-chain polyunsaturated fatty acids, which are indispensable for growth and development [23]. Fatty acids are also recognized to be involved, as gluconeogenic precursors, in the maintenance of normoglycemia in the newborn, an essential mechanism of neonatal adaptation in humans. At birth, the limited carbohydrates stores of the neonate, rapidly depleted, imply the mobilization of fat depots [24, 25]. It was reported that, in human amniotic fluid, free fatty acids (FFA) concentrations are not correlated to maternal plasma FFA, suggesting that amniotic fluid FFA may partially derive from fetus renal excretion [26]. Furthermore, it was demonstrated that, in human pregnancy complicated by IUGR, FFA amount in amniotic fluid is almost three times higher than in normal gestation [27].

The newborn puppy, as the human neonate, is strongly dependent on the limited stores of glucose for normoglycemia maintenance and the risk of hypoglycemia after birth is considered higher in small size breeds when compared with medium and large [28]; thus, fat stores mobilization could be hypothesized also in newborn puppies.

Because of the lack of knowledge about IGF-I and NEFA in canine fetal fluids, and of the possible relationship with neonatal development and breed size, the aims of the present study were as follows:: 1) to evaluate whether or not IGF-I

and NEFA are detectable in amniotic and allantoic fluids collected from normally developed and viable newborn puppies born at term of normal pregnancies; 2) to assess possible differences between IGF-I and NEFA concentrations in amniotic compared to allantoic fluid; 3) because of the wide heterogeneity of body size in dog breeds, the study was also aimed to detect possible relationship between breed body size and IGF-I and NEFA amniotic and allantoic levels; 4) to evaluate possible differences in IGF-I fetal fluids concentrations between male and female puppies; and 5) to assess possible correlations between the two hormones in each type of fluid.

9.3 Materials and methods

9.3.1 Animals and samples collection

The study enrolled 25 owned bitches, 2 to 9 years old, 1 to 7 parity, belonging to several pure breeds and, for this reason, distributed into three different groups according to the breed size: small (bodyweight ≤10 kg, n=16), medium (bodyweight 11-25 kg, n=4), and large (bodyweight 26-40 kg, n=5). Elective Cesarean section was performed in all the cases for health of both mothers and puppies; in fact, these bitches belonged to particular breeds at high risk of dystocia or with a previous history of troubles at parturition. The female dogs were fully monitored from before the mating or artificial insemination (AI), throughout the gestation, until parturition. They showed a body condition score ranging between 2.5/5 and 3/5 and were healthy at a general examination, regularly vaccinated, and submitted to parasites prophylaxis. The bitches were monitored at estrus by vaginal cytology and plasma progesterone (P4) analysis [29], suggesting the single natural mating or AI with fresh semen timed with regard to predicted ovulation. Pregnancy was detected by ultrasounds at 20 to 28 days after the sole mating or AI, when the fetal measurements of the inner chorionic cavity allowed the estimation of parturition date [30]. At the same time, the viability of fetuses was evaluated on the basis of early fetal cardiac motion [31]. During the second half of gestation, all the bitches were fed with commercial foods formulated specifically for pregnant female dogs. At 40 to 45 days of pregnancy, the parturition date was additionally predicted by measuring the biparieal diameter by ultrasonography [30] and the fetal viability was re-evaluated. At the predicted parturition day, the bitches were submitted to elective Cesarean section only if plasma P4 concentrations were ≤2 ng/ml; on

the contrary, when plamatic P4 was still above 2 ng/ml, a daily fetal viability and plasma P4 concentrations check was planned.

Before the surgery, all the owners signed an informed consent, not only for the Cesaeran section, but also specifically to allow the collection of fetal fluids for research purposes.

The anesthesia was performed following the same protocol in all the bitches in order to minimize the negative impact on puppy newborns: premedication with atropine (0.02 mg/kg IM) and metoclopramide (0.2 mg/kg SC), cefazolin (25 mg/kg IV), mask oxygenation, induction with propofol (4-6 mg/kg slowly IV), lidocaine infiltration on the site of surgical incision (2 mg/kg), and maintenance using isofluorane in oxygen. The elective Cesarean section was conventionally performed by a ventral midline laparotomy. As soon as all fetuses were removed, tramadol (3 mg/kg IV) and oxytocin (0.15 IU/kg IM) were injected to the dams.

Fetal fluids were collected from every fetus of each female dog, contemporary at the fetal extraction. A veterinarian, particularly skilled in newborns management, took cares of the puppies, while another one promptly recovered the fluids from each sac at the time of fetal membranes opening, with sterile syringe without needle and only in few instances using it, but avoiding any touch to the newborns. The collected fluids were immediately centrifuged at 1000 x g for 20 minutes and stored at -20° C until analysis for IGF-I and NEFA.

At birth, the neonates were evaluated for normal viability by appearance, pulse, grimace, attitude, respiration score (≥7) [32], maturity, sex, absence of gross physical malformations, and weighted before nursing. Only newborn puppies with a birth weight within the breed reference range reported by the Italian Kennel Club (ENCI) were considered.

9.3.2 IGF-I analysis

IGF-I concentrations in fetal fluids samples were analyzed by RIA after an acid/ethanol extraction to release IGFs from binding proteins. The RIA was performed according to manufacturer's instructions. Insulin-like growth factor-I was determined using an antibody distributed by Novozymes Biopharma (Thebarton, Australia). Recombinant human IGF-I (Novozymes Biopharma) was used as the radioligand and unlabelled ligand. The tracer was prepared with Na 125I by the iodogen method [33]. The minimum detectable dose of IGF-I

was 2.7 pg/tube. Intra- and inter-assay coefficients of variation were 4.4% and 9.1%, respectively.

9.3.3 NEFA analysis

Enzymatic-colorimetric methods were used to determine NEFA concentrations in fetal fluids (Wako Chemicals, Richmond, VA, USA). Intra- and inter-assay coefficients of variation were 2.7% and 5.5%, respectively. Assay sensitivity was 0.140 μEq/l.

9.3.4 Statistical analysis

In this study, the data were presented as mean ± standard deviation (SD). The normal distribution of IGF-I and NEFA concentrations in the different breed size groups (small, medium, and large), genders (male and female puppies), and fluids (amniotic and allantoic) was tested using the Kolmogorov-Smirnov normality test. The significance was assumed for $P<0.05$. The homoscedasticity in different groups was tested using the Levene's test. Because the data were normally distributed in all groups, an ANOVA was performed, considering the bitch size, the puppy gender, and the fluid as a fixed factor, whereas the bitch and the puppy as a random factor. When appropriate, the post-hoc analysis was performed using the Scheffè test. The interactions between the bitch size*fluid type and between gender*fluid type were also tested. A possible correlation between amniotic and allantoic IGF-I and NEFA concentrations was assessed by Pearson correlation test. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and the significance level was set at $P<0.05$.

9.4 Results

9.4.1 Clinical results

The 25 gestations, with normal clinical course, provided a total of 96 newborn puppies at Cesarean section. The neonatal examination evidenced 85 normal neonates, 38 females and 47 males, whereas the remaining 11 newborn puppies were excluded because they were born dead or affected by neonatal malformations (anasarca, cleft palate). Out of 85 normal puppies, a total of 73 amniotic and 76 allantoic samples were analyzed for IGF-I and NEFA concentrations.

9.4.2 IGF-I and NEFA concentrations

The data concerning IGF-I and NEFA concentrations in both amniotic and allantoic fluid were normally distributed. Furthermore, the Levene's test confirmed that all groups were homoscedastic. The mean IGF-I concentration was significantly ($P<0.01$) higher in amniotic (34.2±13.72 ng/ml) than in allantoic (22.8±14.69 ng/ml) fluid, independently by the breed size. Regarding the puppy gender, no significant differences were observed in IGF-I levels between males and females, neither in amniotic (34.4±14.6 ng/ml vs. 34.7±12.9 ng/ml) nor in allantoic fluids (24.3±16.2 ng/ml vs. 20.4±11.5 ng/ml). Taking into account the breed size, it was found that amniotic IGF-I concentrations were significantly lower ($P<0.05$) in small and medium size groups when compared with large size bitches (Table 1), whereas significant differences were not evidenced in allantoic IGF-I levels among size groups (Table 1). A significant interaction was detected between breed size and IGF-I fluid concentration ($P<0.01$). Moreover, a significant effect of the bitch ($P<0.01$), but not of the puppy, on IGF-I levels in both fetal fluids existed. No significant correlations were evidenced between IGF-I concentrations in amniotic and allantoic fluids.

In all the breeds, the mean NEFA concentration did not significantly differ between amnion and allantois. In both amniotic and allantoic fluids, NEFA levels were significantly ($P<0.05$) higher in small breeds when compared with medium and large ones (Table 1). A significant effect of the bitch, or of the puppy, on NEFA concentrations in both fetal fluids was not detected. Significant correlations were not highlighted neither between NEFA levels in amniotic and allantoic fluids nor between IGF-I and NEFA concentrations in amniotic and allantoic fluids.

Table 1: IGF-I and NEFA concentrations (mean ± SD) in amniotic and allantoic fluids of bitches with different body weight.

	IGF-I (ng/ml)		NEFA (µEq/l)	
Bitches (25)	Amniotic fluid (73)	Allantoic fluid (76)	Amniotic fluid (73)	Allantoic fluid (76)
Small: <10 kg (16)	32±13.21[a] (38)	23± 15.01 (41)	44±34.80[a] (38)	38 ± 27.04[a] (41)
Medium: 11-25 kg (4)	29.8 ± 13.19[a] (18)	26.5 ± 18.54 (16)	22.7 ± 10.99[b] (18)	36 ±18[b] (16)
Large: 26-40 kg (5)	43.6 ± 11.46[b] (17)	19.6 ± 9.49 (19)	14.8 ± 10.74[b] (17)	11.9 ± 11.22[b] (19)

[a,b]Values with different superscript letters in the same column differ significantly ($P<0.05$).

9.5 Discussion

In mammals, fetal growth and well-being are regulated by several factors and the placenta contributes to maintain the best environment for the fetus. Fetal fluids, especially the amniotic fluid, should not be simply considered as fetal urines, but instead a marvelously complex and dynamic milieu changing along pregnancy; in fact, it is recognized to contain important factors involved in fetal growth and development, and also in fetal protection [5].

To date, little is known about the composition of amniotic and allantoic fluids in domestic species. Previous studies examined fetal fluids in bovine [34], ovine [35, 36], and, only recently, in cat [37]. On the contrary, in canine species, according to the authors knowledge, the composition of both fetal fluids was not yet fully investigated. For this reason, the present study was designed to provide information about IGF-I and NEFA fetal fluids concentrations in dog. In particular, amniotic and allantoic fluids belonging to mature, viable, and normally developed pure breed newborn puppies were collected, assessing any possible relation to the breed body size.

Despite it should be interesting to study the perinatal amniotic and allantoic composition associated to the changes occurring in both maternal and neonatal

circulations, in the total respect of animal welfare, blood sampling from puppies, and also by the dam, were not performed. The collection of fetal fluids during Cesaeran section represented, therefore, an alternative, not invasive technique for the investigation of prenatal amniotic and allantoic composition.

The present study strongly demonstrated that IGF-I and NEFA concentrations are detectable in both amniotic and allantoic fluids of canine species and that a correlation between these substances and breed size exists, although the number of bitches grouped on the basis of breed size was unbalanced. In fact, the small breeds group was the largest because of the higher susceptibility to dystocia of small breed bitches when compared to medium and large.

In dogs at physiological term pregnancies, IGF-I concentrations appeared higher in amniotic than in allantoic fluid, independently by the breed size. Considering the intimate relationship between the fetus and the amniotic fluid, this finding probably suggests a role of the fetus itself contributing to the amniotic IGF-I concentrations. Additionally, IGF-I levels were significantly higher in amniotic fluids collected from fetuses belonging to large breeds when compared with small and medium. This result seems to demonstrate that amniotic IGF-I could be used as an indicator of growth potential in dogs, as previously suggested in the same species for IGF-I in serum [38], and it is in agreement with the low IGF-I amniotic concentrations reported in human IUGR [19]. Regarding the possible influence of fetal gender, the lack of significant differences in IGF-I levels between male and female puppies in both fluids is in contrast to the higher IGF-I circulating levels detected in human females when compared with male newborns [21]. A significant effect of the bitch on IGF-I concentrations in both fetal fluids was highlighted; in this respect, IGF-I levels vary among puppies belonging to different litters, whereas IGF-I concentrations appear similar among puppies within the same litter. This finding could therefore suggest that the major contribution to IGF-I fetal fluids composition derives from the maternal compartment or from the interaction of maternal and paternal effects, at least when normal maternal and fetal conditions are concerned.

After birth, normoglycemia is very important for the newborn adaptation to the extrauterine life. The newborn puppy, such as the human neonate, must have sufficient stores of glycogen and gluconeogenic precursors, like fatty acids, adequate concentrations of hepatic enzymes for gluconeogenesis and

glycogenolysis, and a functional endocrine system to ensure a normal glucose production. The newborns are strongly dependent on glucose intake but, at birth, their limited carbohydrates stores are rapidly depleted, leading to the mobilization of fat depots [24, 25, 28]. In the present study, no significant differences were found between amniotic and allantoic fluids NEFA concentrations. However, significant differences were noted about NEFA levels in both fluids when breed size was considered; interestingly, NEFA concentrations were higher in amniotic and allantoic fluids belonging to small size dogs than in medium and large breeds, and this result seems to be in agreement with higher NEFA levels reported in human neonates affected by IUGR [27]. Despite the fact that in the present study only normal small size puppies, and not IUGR neonates, were investigated, this finding probably suggests that, also in dogs, NEFA amniotic concentrations could be indicative of fetal, or maternal, or maternal and fetal fat mobilization to cope with the high energetic request at time of parturition. On the contrary to IGF-I, a significant effect of the bitch on NEFA amount in amniotic and allantoic fluids was not detected.

9.6 Conclusions

The present study demonstrates that IGF-I and NEFA are detectable in canine amniotic and allantoic fluids collected at term of pregnancy from normal, mature, and viable pure breed puppies, and that these substances are strongly related to the breed size. Insulin-like growth factor-I could be considered as a possible indicator of growth potential, whereas NEFA as a marker of fat mobilization in response to energy request. Further research is needed to verify the potential relationship between IGF-I and NEFA fetal fluids concentrations and fetal growth pathologic conditions.

9.7 References

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CHAPTER 10

Immunoglobulin G

and

lysozyme

10.1 Introduction

The placenta is a transient organ, typically present during gestation, and responsible for protection, nutrition, respiration, and endocrine control (King, 1982; Chucri *et al.*, 2010). In fact, it is able to transfer several substances from the maternal circulation to fetal one. However, at the same time, carbon dioxide, water, hormones, and residual metabolism products can pass from the fetoplacental circulation to the maternal one. Furthermore, the placenta holds a great immunological role in the antibodies transfer, tolerance and regulation of fetal development, cytokines release, and in the helper and cytotoxic lymphocytes (Michelon *et al.*, 2006; Chucri *et al.*, 2010).

10.2 Transplacental antibodies transfer

In mammals, the passive immunity from maternal antibodies represents a vital component of the immune protection to survive in the extrauterine environment during the first months of age (Tizard, 2009). Indeed at birth, the neonatal immune system is not fully efficient and, however, the immune response to an antigenic stimulation would be a primary, not protective, response.
The transfer of maternal antibodies during pregnancy varies according to species, based on the type of placenta (Enders and Blankenship, 1999; Marques *et al.*, 2007b; Chucri *et al.*, 2010). Both the immune transfer and placental permeability were reported to be inversely proportional to the "placental barrier", that is the number of tissue layers interposed between the maternal and fetoplacental circulation systems (Porter, 1976; Leiser *et al.*, 1994; Chucri *et al.*, 2010). In equines, swines, and ruminants, the placenta, epitheliochorial in the first two species and syndesmochorial in the last one, prevents the antibodies transfer to the fetus during pregnancy, so that the passive immune transfer depends completely on the colostrum intake (Dall'Ara, 2005; Giguère and Polkes, 2005; Morein *et al.*, 2007; Crisman and Scarratt, 2008; Chucri *et al.*, 2010). In endotheliochorial and hemochorial placentation, typical of small carnivores and humans respectively, the infiltration of the trophoblast cells through the uterine epithelium is characteristic. In endotheliochorial placenta of the dog and cat, the uterine endothelium intimately connects with the fetal chorionic villi (Moffett and Loke, 2006; Chucri *et al.*, 2010) and fetomaternal circulation is of simple crosscurrent type (Chucri *et al.*, 2010). Specifically, four cellular layers

separate maternal and fetal circulation systems: the maternal uterine endothelium, chorion, fetal mesenchyme, and fetal endothelium (Miglino *et al.*, 2006; Chucri *et al.*, 2010). Placental hematomas represent another important feature of the endotheliochorial placenta, since in these areas the phagocytosis of fetal cells occurs from elements of maternal blood (Amoroso, 1952). Additionally, Enders and Carter (2004) localized the iron transfer in carnivores hematophagous areas. Stoffel *et al.* (1998) reported that the canine placenta showed also the endotheliochorial labyrinth of placental belt and the placental free zone, considered as two other areas of transplacental exchanges.

In comparison to humans, in which a significant amount of immunoglobulin is transferred transplacentally, the type of placentation in canine species results in very little maternal immunoglobulin transfer to the fetus, with reported transplacental immunoglobulin passage ranging from 5% to 10% (Tizard, 2009; Chucri *et al.,* 2010; Evermann *et al.*, 2011).

The placental transport of immunoglobulin was deeply investigated in humans. In this species, only the class of immunoglobulin G (IgG) is transferred from mother to fetus (Simister, 2003; Kane and Acquah, 2009). Immunoglobulin G concentrations in fetal blood rise from early in the second trimester to the term of gestation, since the most antibodies passage occurs during the third trimester (Simister, 2003). The timing of the beginning in immunoglobulin transport across the placenta is still controversial. Maternofetal transfer of radiolabeled IgG was minimally detected at around 12 weeks gestation, in agreement with low IgG levels present in the villous stroma of placenta at 8-10 weeks pregnancy (Bright and Ockleford, 1995; Simister, 2003). Several studies documented an increase in fetal IgG concentrations in the second trimester; particularly, more recent researches revealed a smooth rise from 13-18 weeks pregnancy (Garty *et al.*, 1994; Malek *et al.*, 1996; Simister, 2003). According to Simister (2003), the immune transfer starts at 16 weeks gestation, whereas Saji *et al.* (1999) asserted that the passage through placenta begins slowly at approximately 20 weeks pregnancy. Anyway, fetal IgG levels continue to increase along the third trimester (Garty *et al.*, 1994; Malek *et al.*, 1994; Malek *et al.*, 1996; Simister, 2003; Chucri *et al.*, 2010). The greatest rate of antibodies transfer to fetus occurs after 34 weeks gestation, so that premature newborns do not receive fully protective levels of antibodies (Landor, 1995). In women, during the last trimester of pregnancy, transported IgG crosses the syncytiotrophoblast covering the chorionic villi and the fetal capillaries endothelium. It was evidenced that the

neonatal Fc receptor, FcRn, mediates the IgG transmission across the syncytiotrophoblast (Moraes-Pinto *et al.*, 2001; Radulescu *et al.*, 2005; Simister, 2003). FcRn is expressed in the syncytiotrophoblast by late in the first trimester, but at this developmental stage the syncytiotrophoblast covers a continuous cytotrophoblast layer. Cytotrophoblast cells may prevent further villi penetration by IgG, as they neither express FcRn, nor contain IgG. As pregnancy progresses, the syncytiotrophoblast expands, whereas the cytotrophoblast becomes discontinuous; thus, the surface area for IgG uptake from maternal blood is increased and this also allows the access to the fetal vessel endothelium. The mechanism of IgG transport across the endothelium of fetal capillaries remains to understand. Normally at term, human fetal IgG exceeds maternal levels (Longsworth *et al.*, 1945; Kohler and Farr, 1966; Simister, 2003). IgG1 represents the most efficiently transported subclass, whereas IgG2 the least one (Garty *et al.*, 1994; Malek *et al.*, 1996; Malek *et al.*, 1996; Simister, 2003). It was demonstrated that low birth weight neonates have reduced circulating IgG concentrations compared to those of similar gestational age with normal birth weight (Catty *et al.*, 1979; Simister, 2003), since probably placental insufficiency limits IgG transport (Sierig *et al.*, 2002). Additionally, according to Bianco *et al.* (1983), mean IgG levels in the human amniotic fluid resulted higher in case of infection compared to those in normal amniotic fluid.

The mechanism of transplacental passive immunity in dogs was not yet clearly illustrated. However, it is already known that IgG is the primary antibody transferred, as this antibody was detected in fetal capillaries (Stoffel *et al.*, 2000). Early researches reported both prenatal and postnatal antibodies transfer in canine species, nevertheless the morphological evidence of the transplacental transport route was investigated more recently by Stoffel *et al.* (2000). In the bitch, some antigenic-specific passive immunity is conferred to fetuses during the last third of pregnancy. The presence of IgG was clearly demonstrated in all the layers of the materno-fetal barrier in the labyrinth zone. Immunoreactivity appeared particularly prominent in maternal basement membrane material, as well as in the syncytiotrophoblast. In the haemophagous zone, IgG was very abundant in cytotrophoblast cells, but not detectable in the underlying mesenchyme and fetal capillaries. Since maternal antibodies seem to be degraded in the cytotrophoblast cells of the haemophagous zone, materno-fetal transfer of IgG is likely restricted to a subpopulation of maternal vessels in the labyrinth

zone. However, further studies are needed to clarify the mechanism of transport and its restriction to individual vessels (Stoffel *et al.*, 2000).

10.3 Lysozyme

Lysozyme is an hydrolytic enzymes, able to catalyse the hydrolysis of the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer (Ogundele, 1998; Callewaert and Michiels, 2010). Thus, one of its recognized function is the antibacterial defence, as it lyses mostly Gram-positive and a few Gram-negative bacteria, or induces their aggregation (Witholt *et al.*, 1976). Among the anti-inflammatory functions, lysozyme was shown to inhibit the chemotaxis of activated leucocytes, mitogen-induced lymphoblastogenesis, and autologous mixed lymphocyte reaction (Gordon *et al.*, 1979). Furthermore, Ogundele (1998) reported its ability to inhibit the haemolytic activity of serum complement, highlighting a new anti-inflammatory action of this substance. Finally, in some animals, lysozyme keeps even a digestive role.

In humans, c-type lysozyme was detected in several body fluids (tears, saliva, airway fluid, breast milk, urine, serum, cerebrospinal fluid, cervical mucus, and amniotic fluid), in tissues, including the respiratory and intestinal tracts, and in the lysosomal granules of neutrophils and macrophages (Callewaert and Michiels, 2010).

As above mentioned, lysozyme was found also in human amniotic fluid (Schlievert *et al.*, 1977; Barling *et al.*, 1985), where it works mainly as one of the antimicrobial system factors (Schlievert *et al.*, 1977; Mega *et al.*, 1981). Amniotic lysozyme was supposed to originate from several source, such as the placenta itself, saliva, urine, and fetal cord serum (Umezu *et al.*, 1985). In human amniotic fluid, its content was suggested to resemble index of fetal maturity; indeed, normal amniotic fluid lysozyme level increases gradually from the early stage of gestation to the late part of the second trimester, and rapidly after 32 weeks (Hisanaga *et al.*, 1982). In addition, Porto *et al.* (1990) reported that lysozyme in the amniotic fluid could be an indicator of fetal distress.

Seeing the placental type in dogs, colostrum and milk represent the major sources of immunity, thanks to both specific and non-specific factors. At this regard, Rota *et al.* (2008) documented the presence of IgG and lysozyme in peri-partum mammary secretions of the bitch. Interestingly, whereas IgG class was

high at the beginning and declined significantly 1-2 days after whelping, lysozyme tended to remain high until 5 days after parturition. Another study (Schäfer-Somi *et al.*, 2005), assessing the immunoglobulin presence in nasal secretions of canine newborns, demonstrated the predominance of IgG during the first 3 days of age. These findings highlighted the importance of the early colostrum adsumption after birth for IgG absorption, whereas the prolonged presence of lysozyme ensured an extended functional non-specific immunity for the puppy, as reported in other mammals (Sarwar *et al.*, 2001). The lysozyme levels were investigated also in mammary secretions of cows (Król *et al.*, 2010), donkey jennies (Veronesi *et al.*, 2014), and mares (Sarwar *et al.*, 2001).

Because of the lack of knowledge about IgG and lysozyme content in canine fetal fluids, the aims of the present study were to assess the detectability of these substances in both amniotic and allantoic fluids, collected by full developed and viable puppies born at term of normal pregnancies, evaluating IgG and lysozyme concentrations in both fluids and any possible correlation between their levels and some maternal and neonatal factors.

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CHAPTER 11

Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy

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11. Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy

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11.1 Abstract

In the dog, the endotheliochorial placenta allows only the 5 to 10% transfer of maternal antibodies to the fetus, but the timing and the factors influencing the immunoglobulin G (IgG) transplacental transport were not fully investigated. The aims of the present study were the following: 1) to assess the presence of both IgG and lysozyme in amniotic and allantoic fluids collected from fully developed and viable newborn puppies born by elective Cesarean section at term, and possible correlations between amniotic and allantoic IgG and lysozyme levels; 2) to verify possible differences in IgG and lysozyme concentrations between the two fluids; and 3) to detect possible differences in IgG and lysozyme fetal fluids levels in relation to the maternal breed body size and parity, as well as to the neonatal gender. The study, performed on 41 purebred bitches submitted to elective Caesarean section at term, enrolled 142 puppies, 74 males and 68 females, born mature, viable, without gross malformations, and with a normal weight. At surgery, a total of 129 amniotic and

84 allantoic samples were collected for IgG and lysozyme analysis. Class G immunoglobulins and lysozyme were detected in both fluids, but IgG concentrations were higher ($P<0.01$) in amniotic fluid. Moreover, a significant positive correlation ($P<0.01$) between IgG amniotic and allantoic levels, but not for lysozyme, was observed. A significant effect of the maternal parity ($P<0.05$), but not of the breed body size, on the amniotic IgG concentrations was found, whereas the newborn gender was not associated to different IgG or lysozyme amniotic and allantoic levels. Given the significant contributions of fetal fluids to fetal and neonatal health, the results reported that the amniotic and allantoic fluids play a role in the immune protection of the fetus/newborn also in canine species. However, additional research is needed to better elucidate both the origin of IgG and lysozyme and the factors influencing the wide interindividual variations.

11.2 Introduction

Immediately after birth, the newborn is highly susceptible to infectious diseases because of the immune system immaturity and sensitivity to tolerogenic signals. Although the adaptive immune system is not completely developed and the full immunologic competence is normally reached several weeks after birth, the newborn should cope with the new harmful extrauterine environment, where several factors are frequently responsible for neonatal morbidity and mortality [1]. Therefore, the neonatal immunologic protection depends on the immunity passively acquired by the mother through the placenta or colostrum, or both [2].

In eutherian mammals, the placenta is a transient organ, responsible not only for the mechanical protection, nutrition, respiration, and endocrine control of the fetus during pregnancy [3], but also for the immunologic defense guaranteed by the antibodies transfer. The canine placenta is classified as endotheliochorial [4], so that the uterine endothelium connects intimately with the fetal chorionic villi. Furthermore, the maternal and fetal circulation systems are separated by four tissue layers: the maternal uterine vessels endothelium, chorion, fetal mesenchyme, and fetal endothelium [5]. In the bitch, only 5 to 10% of maternal antibodies is transferred to the fetus, whereas the transit of the remainder passive immunity is guaranteed after birth by colostrum [6]. The mechanism of transplacental passive immunity in canine species is still not completely clarified. However, the finding of the immunoglobulins of class G (IgG) within the fetal

capillaries [7] demonstrated that IgG represent the primary antibodies transferred via the placenta in the dog. In addition, in the newborn puppy, the serum IgG concentration is documented to be around 5 to 10% of the adult level [8].

Contrary to humans, in which both the timing and factors influencing the transplacental transport of IgG were widely studied [9], in canine species the knowledge about these topics are very scarce. The literature reports that IgG placental transfer in humans depends on several factors, such as the maternal levels of immunoglobulins and parity, gestational age, maternal vaccination during pregnancy, and neonatal birth weight [9, 10]. It was suggested that the IgG levels in the newborn usually are well correlated with the maternal ones; nevertheless, the amount of IgG transferred was reported to depend on the number of cell surface specific receptors (FcRn) [9]. Concerning the possible influence of the neonatal gender, at the authors knowledge, it was not associated to different IgG placental transport in human beings, even if amniotic angiogenin levels were found significantly lower in amniotic fluid samples collected from pregnancies with a male than those with a female fetus [11].

The immune system includes also the action of several nonspecific factors, such as lysozyme. This important hydrolytic enzyme is particularly able in the lysis of peptidoglycan within the bacterial cell wall, especially against Gram-positive bacteria. Lysozyme was detected in many human biological samples, among which the amniotic fluid [12-14] where it shows mainly antimicrobial and antiinflammatory properties. Amniotic lysozyme was supposed to originate from several source, such as the placenta itself, saliva, urines, and fetal cord serum [15], and its amount was suggested to be an index of fetal maturity [16]. Unfortunately, to the authors knowledge, the presence as well as the concentrations of lysozyme in dog fetal fluids were never investigated.

Because the information about IgG and lysozyme content in canine fetal fluids are lacking, the aims of the present study were to 1) assess the detectability of IgG and lysozyme in amniotic and allantoic fluids collected by fully developed and viable puppies born at term of normal pregnancies, and possible correlations between amniotic and allantoic IgG and lysozyme levels; 2) verify possible differences in IgG and lysozyme concentrations between the two fluids; and 3)

detect possible differences in IgG and lysozyme fetal fluids levels in relation to the maternal breed body size and parity, as well as to the neonatal gender

11.3 Materials and methods

11.3.1 Animals and samples collection

The study was performed in the year 2012, when the national regulations did not require an istitutional approval for the use of waste biological material obtained during routine clinical management of patients. Fourty-one owned bitches, 2 to 10 years old, 11 primiparous and 30 multiparous, were enrolled. Because the female dogs belonged to five different breeds, they were grouped on the basis of breed size: small (bodyweight ≤10 kg, n=30) and medium-large (bodyweight >10 kg, n=11). Particularly, the first group included Chihuahua and Maltese, whereas the second group enrolled Shar-Pei, Maremma, and Leonberger. All the bitches underwent the elective Cesarean section because of the high risk of dystocia of the breeds enrolled or previous history of troubles at parturition. These female dogs were monitored from before the mating, throughout the gestation, until whelping. They were healthy at a general examination, regularly vaccinated and submitted to parasites prophylaxis. The last vaccination occurred between one and two months before the expected date of mating. Only four dogs were vaccinated during pregnancy against Canine Herpes Virus, according to the manufacturer instructions. The breeding time determination, as well as the pregnancy detection and monitoring, were performed as previously reported [17]. At the predicted parturition day, the bitches underwent the elective Cesarean section only if plasma P4 concentrations were 2 ng/ml or lesser. On the contrary, when P4 in plasma was still above 2 ng/ml, a daily fetal viability and plasma P4 levels check was planned.

Before the surgery, all the owners signed an informed consent, both for the Cesarean section and the fetal fluids collection for research purposes.

The same anesthetic protocol was used in all the bitches in order to minimize the negative impact on puppy newborns viability. Cesarean section was performed by celiotomy, as reported by Meloni *et al.* [17], and fetal fluids were collected from every fetus of each female contemporary at fetal extraction. Specifically, the fluids were recovered from each sac at the time of fetal membranes opening. First, the allantoic sac was minimally sliced and the allantoic fluid was collected

with a sterile syringe without needle; second, the amniotic sac was opened and the incision edges were hold to avoid the possible contamination and allow the collection of the amniotic fluid, always by using a sterile syringe without needle. In case of fluids mixture, or even when whatever contamination was suspected, the samples were not included in the study. The collected fluids were immediately centrifuged at 1000 x g for 20 minutes and stored at -20° C until analysis for IgG and lysozyme, for a time not exceeding 3 months. The collection of the fetal fluids absolutely did not modify neither the course of surgery nor the newborn care, since a single operator was totally devoted to the fluids collection.

At birth, the neonates were evaluated for maturity, viability by APGAR score [18], absence of gross physical malformations, and gender, and weighted before nursing. Body weight at birth was compared to the breed reference range reported by the Italian Kennel Club (ENCI).

11.3.2 IgG analysis

Immunoglobulins G concentrations in amniotic and allantoic fluid were analyzed by an ELISA catching (Dog IgG Quantitation Set; Bethyl Laboratories, Inc., Montgomery, TX, USA). Ninety-six-well microtitre plates were coated for 1 hour at room temperature with 100 μl/well of capture affinity purified antibody (sheep anti-dog IgG) diluted 1:100 in coating buffer (carbonate-bicarbonate buffer, 0.05 M; pH 9.6); at the end of the incubation, the plates were washed five times with Tris buffered saline (TBS) of 50 mM, pH 8.0 with 0.05% Tween 20 (TBST). Successively, the plates were postcoated with 200 μl/well of TBST for 30 minutes, and then washed again five times. Seven twofold dilutions of reference dog antibodies were used (from 500-7.8 ng/ml). On the basis of many set up assays (performed to evaluate the optimal dilutions of both fluids depending on the expected results), the amniotic and allantoic fluids were diluted differently; the amniotic fluids were diluted 1:5000, whereas the allantoic fluids were diluted 1:500. All dilutions were prepared in TBST. In each well, 100 μl of each reference serum or sample dilution were added in duplicate and incubated for 1 hour at room temperature, and then washed five times before adding 100 μl/well of conjugate (sheep anti-dog IgG horseradish peroxidase [HRP] conjugate, diluted 1: 100000), the latter incubated for 1 hour at room temperature. The wells were washed five times before adding 100 μl/well of

substrate-chromogen (H_2O_2 and 3,3',5,5'-tetramethylbenzidine [TMB]), left for 15 minutes in the dark; the reaction was stopped by adding 100 µl/well of stop solution (sulfuric acid, 0.18 M), and the plates were read at 450 nm with an ELISA microplate reader (Titertek Multiskan, Labsystem), expressing the result as optical density.

11.3.3 Lysozyme analysis

Lysozyme concentration was assayed using a micromethod assay [19], starting from the classic lysoplate method [20]. A suspension of *Micrococcus lysodeikticus* cell walls (Sigma) at a final concentration of 0.6 mg/ml in 1% low electroendosmosis and sulfate content agarose prepared in 0.066-M phosphate buffer, pH 6.6 was poured in aliquots of 50 ml into 15-cm diameter Petri dishes. Just before use, 44 wells, 3-mm diameter, were punched into the gel with a cork borer and evacuated with a Pasteur pipet connected to a water aspirator. Samples and standard solutions (10-5-2.5-1.25-0.625 µg/ml) from chicken egg white lysozyme (Sigma), prepared in the same buffer used for the gel medium, were placed in the wells in duplicate (10 µl/well). After plates incubation at 24°C for 18 hours, the diameter of the cleared zone of lysis was measured with a special ruler. Exponential standard curves from the size of the cleared zones of the lysozyme standard solutions, from which the lysozyme concentration in the unknown samples was estimated, were used.

11.3.4 Statistical analysis

Data are shown as mean ± standard deviation (SD). The normal distribution of IgG and lysozyme concentrations in the two types of fluid (amniotic and allantoic), according to the maternal breed body size (small and medium/large) and parity groups (primiparous and multiparous), as well as in the newborn gender groups (male and female), was tested using both the Kolmogorov-Smirnov and the Shapiro-Wilk normality tests. A possible correlation between amniotic and allantoic IgG and lysozyme levels was assessed by the Spearman correlation test. Because the data were not normally distributed in all tested groups, the homoscedasticity in different groups was tested using the nonparametric Levene test, followed by an ANOVA test, considering the type of fluid, the bitch size, and the gender of puppy as a fixed factor, whereas both the bitch and puppy as a random factor. The interactions between the bitch size and fluid type as well as between the puppy gender and fluid type were also tested.

Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). In all cases, the significance level was set at $P<0.05$.

11.3 Results

11.4.1 Clinical findings

At the end of the 41 normal pregnancies, 142 puppies were born mature, viable, without gross malformations, and with a normal weight, by Cesarean section; three additional puppies were excluded because they were born dead. Of 142 normal puppies, 74 males and 68 females, a total of 129 amniotic and 84 allantoic samples were collected for IgG and lysozyme analysis.

11.4.2 IgG and lysozyme concentrations

Both IgG and lysozyme were present and detectable in amniotic and allantoic fluids. However, IgG concentration was significantly higher ($P<0.01$) in the amniotic as compared with the allantoic fluid (0.14 ± 0.17 mg/ml vs. 0.06 ± 0.06 mg/ml, respectively), whereas a significant difference was not evidenced in the mean level of lysozyme between amniotic and allantoic fluids (Table 1). Regarding the possible correlation between the two fluids, the Spearman rho test showed a statistically significant positive correlation ($P<0.01$) between the IgG concentrations in amniotic and allantoic fluids, but not between the lysozyme levels in the two fluids.

Table 1: IgG and lysozyme concentrations (mean ± SD) in amniotic and allantoic fluids belonging to the 41 bitches.

IgG (mg/ml)		**Lysozyme (μg/ml)**	
Amniotic fluid (129)	Allantoic fluid (84)	Amniotic fluid (129)	Allantoic fluid (84)
0.14 ± 0.17^{a}	0.06 ± 0.06^{b}	1.9 ± 1.76	3.1 ± 1.58

[a,b]Values with different superscript letters in the same line differ significantly ($P<0.01$).

When amniotic and allantoic IgG and lysozyme concentrations were analyzed in relation to maternal breed body size, a significant difference was not found neither for IgG nor for lysozyme (Table 2).

Table 2: IgG and lysozyme concentrations (mean ± SD) in amniotic and allantoic fluids of bitches with different body size.

Bitch body size	**IgG (mg/ml)**		**Lysozyme (µg/ml)**	
Bitches (41)	Amniotic fluid (129)	Allantoic fluid (84)	Amniotic fluid (129)	Allantoic fluid (84)
Small: <10 kg (30)	0.15 ± 0.19 (88)	0.06 ± 0.05 (60)	1.9 ± 2.07 (88)	3.1 ± 1.75 (60)
Medium-large: >10 kg (11)	0.1 ± 0.1 (41)	0.06 ± 0.07 (24)	1.9 ± 0.77 (41)	2.9 ± 1.03 (24)

On the contrary, IgG level was significantly higher ($P<0.05$) in amniotic fluid of pluriparous bitches as compared with primiparous ones (0.15 ± 0.18 vs. 0.07 ± 0.05 mg/ml, respectively). This difference was not observed for lysozyme concentrations neither in amniotic nor in allantoic fluid (Table 3).

Table 3: IgG and lysozyme concentrations (mean ± SD) in amniotic and allantoic fluids of bitches related to parity.

Parity	**IgG (mg/ml)**		**Lysozyme (µg/ml)**	
Bitches (41)	Amniotic fluid (129)	Allantoic fluid (84)	Amniotic fluid (129)	Allantoic fluid (84)
Primiparous (11)	0.07 ± 0.05[a] (24)	0.06 ± 0.07 (23)	1.6 ± 0.75 (24)	2.8 ± 0.87 (23)
Multiparous (30)	0.15 ± 0.18[b] (105)	0.06 ± 0.05 (61)	2 ± 1.91 (105)	3.2 ± 1.77 (61)

[a,b]Values with different superscript letters in the same column differ significantly ($P<0.05$).

In addition, IgG and lysozyme levels in both fluids did not differ significantly when newborn gender was considered (Table 4).

Table 4: IgG and lysozyme concentrations (mean ± SD) in amniotic and allantoic fluids of bitches related to fetal gender.

Fetal gender	**IgG (mg/ml)**		**Lysozyme (μg/ml)**	
Fetuses (142)	Amniotic fluid (129)	Allantoic fluid (84)	Amniotic fluid (129)	Allantoic fluid (84)
Males (74)	0.14 ± 0.16 (66)	0.06 ± 0.06 (47)	1.8 ± 0.73 (6)	3 ± 1.34 (47)
Females (68)	0.13 ± 0.17 (63)	0.05 ± 0.05 (37)	2 ± 2.41 (63)	3.1 ± 1.85 (37)

11.4 Discussion

To the authors knowledge, this is the first study in which IgG and lysozyme were investigated in dog fetal fluids collected from mature, viable, and normal newborn puppies, born by elective Cesarean section at term. The present research reported that both IgG and lysozyme are detectable in canine amniotic and allantoic fluids at term pregnancy, suggesting that, also in the dog, both fluids provide fetal protection, not only by mechanical cushioning but also as a part of the innate immune system, which includes antimicrobial effectors, such as lysozyme, lactoferrin, α-dephensin, and so forth [21]. The amniotic fluid is known to play a wide variety of roles to allow the correct development and growth of a healthy fetus. Normally, both maternal and fetal compartments concur in amniotic fluid composition, whereas allantoic fluid could be considered as fetal urine. Thus, all the compounds found within the allantoic fluid presumably result from the fetal excretion; on the contrary, the substances detected in the amniotic fluid could originate from the maternal, fetal, or both compartments.

A high interindividual variation was found for amniotic and allantoic fluids, as suggested by the wide standard deviations, even within each litter, especially for

the amniotic IgG. This high interindividual variation could be due to a multiplicity of causes, including the maternal concentrations of immunoglobulins and the rate of transplacental transfer, as reported for the final levels of antibodies in the human neonatal blood [22].

Despite the different type of placenta between dogs and humans, the amniotic IgG mean concentration (0.14 mg/ml) was similar to that documented for the women amniotic fluid (0.11-0.17 mg/ml) [23]. Additionally, like in humans, this IgG level represents about 1/100 of the serum concentration of adult individuals (10-20 mg/ml in dogs and 8-18 mg/ml in humans). In the human amniotic fluid, beyond the predominant maternal IgG, different molecular forms of fetal immunoglobulins were detected [24]. In the present study, it was not possible to report the exact origin of the amniotic IgG. Thus, it may be speculated that, even in dogs, the final amount of amniotic IgG could derive from a cumulative contribution of both maternal and fetal counterparts. Because in women the IgG placental transfer depends, among several factors, also by the maternal levels of immunoglobulins, the evaluation of circulating IgG in the bitches would have been interesting. Unfortunately, despite all bitches were vein-cannulated during surgery, the analysis of maternal blood IgG and lysozyme was not performed because most of the owners did not agree for the use of a maternal blood sample for research purposes. Thus, it was not possible to better elucidate the real maternal contribution on amniotic IgG and lysozyme. In this study, IgG were detected also in the allantoic fluid, although at significantly lower concentrations (about one half) in comparison with the amniotic fluid. This finding could, on one hand, be explained by a direct fetal production of IgG, but, on the other hand, the presence of IgG in the fetal urine could be merely due to the functional immaturity of the kidney in the canine fetus. In fact, the renal glomeruli are still developing and permeable at large molecules like antibodies, that can therefore pass through the kidney in the urine and then in the allantoic fluid [25].

Beyond other essential substances, the fetal fluids contain lysozyme (mean 1.9 and 3.1 μg/ml in the amniotic and allantoic fluid, respectively), in concentrations similar to the lower levels reported in the serum of adult dogs (2-13 μg/ml; unpublished data). Also for lysozyme, it could be supposed a maternal, fetal, or materno-fetal origin. Even if more homogeneous levels (as evidenced by the narrow standard deviations) were found in the allantoic as compared with

amniotic fluid, no significant differences were detected in lysozyme concentrations between the two fluids. Several studies highlighted the presence of a multitude of innate antimicrobial peptides in the vernix caseosa and amniotic fluid in humans [26]. In women, these antimicrobial peptides act as important immunomodulators, working synergistically to provide a first defense line and promote an interaction between the adaptive and innate immune system in the newborn [27]. It is reasonable to suppose that, even in the dog, lysozyme could be active in fetal fluids similarly to what suggested for humans, providing a first line of defense against infectious agents.

No significant differences were found between IgG or lysozyme amniotic or allantoic concentrations and maternal breed body size. This is in agreement with what observed in humans, because a relation between amniotic IgG levels and race/ethnicity was not reported. In a study performed by Kennedy *et al.* [28], considering the immune response to antirabies vaccination in dogs, small size breeds were considered "high responders", in comparison with large size breeds.

It is interesting to note the finding of significantly ($P<0.05$) higher IgG amniotic concentrations in samples collected from multiparous compared with primiparous bitches, which further reinforce the hypothesis of a major maternal contribution to the final amniotic IgG amount. This result appears in disagreement with the lack of correlation between maternal parity, and age, and amniotic fluid IgG levels reported for humans [9, 10].

A possible effect of the fetal gender on amniotic and allantoic IgG and lysozyme concentrations was not highlighted, confirming that, also in canine species, both fluids belonging to female or male newborns have similar immunoglobulins and antimicrobial lysozyme levels.

A final consideration concerns the four dogs undergone the vaccination against Canine Herpes Virus during pregnancy. Although the statistical analysis on the possible differences in IgG (and lysozyme) concentrations between the fetal fluids collected by vaccinated and nonvaccinated bitches was not performed because of the paucity of samples belonging to vaccinated subjects (11 amniotic and allantoic samples), just at a descriptive analysis, the IgG levels resulted very similar in the two groups of samples (mean 0.04 vs. 0.06 mg/ml in samples from vaccinated and nonvaccinated bitches, respectively). In humans, it was documented that, even if several vaccines are given to pregnant women to

guarantee the fetal protection during gestation or the neonatal protection after birth, many factors may limit the efficacy of antibodies placental transfer after maternal vaccination [9].

In conclusion, the results of the present study reported that the amniotic and allantoic fluids play a role in the immune protection of the fetus/newborn also in canine species. Class G immunoglobulins and lysozyme were detected in both amniotic and allantoic fluids, but IgG levels were higher in amniotic fluid. A significant effect of the maternal parity, but not of the breed body size, on the amniotic IgG concentrations was observed, whereas the newborn gender was not associated to different IgG or lysozyme amniotic or allantoic levels. Given the significant contributions of fetal fluids to fetal and neonatal health, additional research is needed to better elucidate both the origin of IgG and lysozyme and the factors influencing the wide interindividual variations.

11.6 References

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CHAPTER 12

Cortisol levels in hair and nails of newborn puppies

12.1 Introduction

The late pregnancy and the neonatal period represent two most stressful stages, respectively for mammals fetus and newborn. In canine species, natimortality and neonatal losses account to up 30%, so that a full knowledge about the fetal development physiology during the last intrauterine phase, as well as about the process of birth and neonatal adaptation, seems to be necessary to reduce the impact of canine neonatal death. However, unlike other domestic animals species, these topics remain still not completely clarified in dogs.

12.2 Cortisol assessment in biological matrices

Toward the end of gestation, the fetal HPA axis activation of the subject occurs and, in response to adrenocorticotrophic hormone, the adrenals release cortisol (C), beyond other hormones which were recognized the major steroids produced by the fetus itself (Gitau *et al.*, 2001). During the last days of pregnancy, C is involved in multi-organs final maturation (Bolt *et al.*, 2001) and trigger of parturition process, whereas during the first month of age it is released in response to some important neonatal physiologic changes and gradual adaptation to the extrauterine life (Thorburn *et al.*, 1972; Fowden, 1995).

Cortisol is the most common glucocorticoid in humans, non-human primates, and many larger mammals, in which it is considered the primary end point stress hormone. In fact, it was judged a perfect biomarker of acute or chronic stress and a useful tool for long-term monitoring of HPA axis activity in both humans and animals (Russell *et al.*, 2011). This ability to reflect stress levels over long periods of time was limited probably because of the nature of the traditional matrices in which the hormone levels were measured. Indeed, in the most studies, C concentrations were regularly assessed through serum, saliva, and urine. Both serum and saliva samples provide an instantaneous view of the C levels; therefore, they can be used to test only acute changes, since are subject to major physiological daily fluctuations. Total serum C includes both protein-bound and free cortisol; consequently, it could result affected in case of changes in the levels of cortisol-binding globulin. Instead, in saliva only unbound and bioactive hormone can be measured and the collection is less invasive. Moreover, both serum and saliva samples require specific procedures for the storage. On the contrary, urine provide a short-term view of C levels, which

follow a particular diurnal rhytm. Urinary C reflects only the free portion of the hormone and requires the refrigeration or freezing of the sample (Russell *et al.*, 2011; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012; Maidana *et al.*, 2013).

12.3 Hair and nails for hormonal measurement

In humans and animals, hair represents the newest biological matrix in which hormone levels measurement is possible. For decades, hair analysis was used for monitoring exposure to exogenous compounds, overall drugs of abuse. More recently, there was a growing interest in quantifying endogenously produced compounds (Gow *et al.*, 2010). Cortisol concentrations analysis in hair samples is a non invasive technique and it allows to have a long-term retrospective picture of the hormone previous accumulation; in fact, hair C results by the accumulation and incorporation from plasma over a period of months before sample collection. Additionally, hair C levels are not affected by circadian hormonal variations or by factors inducing short-term changes (Russell *et al.*, 2011; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012; Maidana *et al.*, 2013).

Cortisol is supposed to enter the hair at the shaft medulla through passive diffusion from blood. This diffusion is amplified by high lipid solubility and low protein binding, suggesting the preferential deposition of unbound cortisol. Additional C may coat the outer cuticle from sebaceous and eccrine secretions (Raul *et al.*, 2004; Pragst and Balikova, 2006). In a recent study, Russell *et al.* (2014) documented that human sweat contains C concentrations comparable with saliva, thus perfuse sweating after intense exercise may increase hormone levels detected in hair. Whether the hair C is representative of systemic concentrations remains under discussion. Most authors suggested that hair C content is representative of systemic levels, even if local hormone production may participate; indeed, it was demonstrated that hair follicles contain a functional equivalent of the HPA axis and can synthesize C after stimulation by CRH (Ito *et al.*, 2005).

Hair C measurement was reported as validated method in humans (Raul *et al.*, 2004; Sauvé *et al.*, 2007; Kirschbaum *et al.*, 2009; Gow *et al.*, 2010, D'Anna-Hernandez *et al.*, 2011), grizzly bears (Macbeth *et al.*, 2010), rhesus monkeys (Davenport *et al.*, 2006), cows (Comin *et al.*, 2011; Comin *et al.*, 2013; Del Rosario

Gonzàlez-de-la-Vara *et al.*, 2011), horses (Comin *et al.*, 2012), dogs, and cats (Accorsi *et al.*, 2008), even if the number of studies in small animals is absolutely lower than those performed in humans.

During gestation it was demonstrated that a strong maternal stress can negatively affect fetal HPA axis regulation (Van den Bergh *et al.*, 2007) and that abnormal patterns of C are associated with consequent miscarriage, increased fetal activity, premature birth, and decreased birth weight in women (Nepomnaschy *et al.*, 2006; Field and Diego, 2008). Interestingly, D'Anna-Hernandez *et al.* (2011) reported a gradual increase of C amounts during human pregnancy, highlighting the correlation between hair and salivary C levels in pregnant and post-partum women. Yamada *et al.* (2007) evaluated hair C concentrations in newborns, founding higher values in hospitalized neonates than in healthy ones, probably due to their more stressful condition.

In a recent study on bovine species, hair C levels were analysed in clinically or physiologically compromised cows, as well as in clinically normal lactating cows at least two month after calving. The findings demonstrated a significant positive correlation between hair C and severity of clinical condition, and significant differences between hair C levels recorded in each disease subgroup compared with clinical healthy cows (Comin *et al.*, 2013).

Cortisol concentrations were assessed even in equine hair. Samples collected from normal, healthy foals at birth, 30 and 60 days of age, revealed a significant decline of C levels from birth to two months of age, with high inter-individual variations, underlining a progressive adaptation of the newborn to the extrauterine environment (Comin *et al.*, 2012). Silver *et al.* (1984) reported low plasma C concentrations in premature foals, thus analysis of hair samples could be interesting in case of prematurity.

To date, little is known about the hair C detection in canine species, even less in cats. In dogs Bryan *et al.* (2013) evidenced that a single hair sample is better than multiple samples of saliva or faeces to evaluate long-term C levels; in this study no correlations were found neither between hair and faeces nor between hair and saliva, in contrast to previous papers (Accorsi *et al.*, 2008; Bennett and Hayssen, 2010). In canine species, a difference in C concentrations was detected on the basis of different coat colour: particularly, black dogs had less C in hair than non black dogs and, within the same subject, black hair was lower in C than

not black one. In contrast to humans (Kirschbaum *et al.*, 2009), the average amount of C did not differ between proximal and distal hair sections (Bennett and Hayssen, 2010), probably due to the different hair length and hygiene. To date, the possible influence of gender on the hormonal concentrations was investigated in plasmatic samples, suggesting higher plasma C levels in adult male dogs than in female ones (Mongillo *et al.*, 2014), whereas the previous literature reported no sex-related differences in basal concentrations in canine species (Reimers *et al.*, 1984; Garnier *et al.*, 1990; Reimers *et al.*, 1990; Hennessy *et al.*, 1997). Previous researches documented that basal C levels appeared to be influenced also by breed body size and age (Reimers *et al.*, 1990). Indeed, smaller breeds tended to have higher basal C concentrations than larger breeds, as well as nursing pups had higher levels than older dogs (Reimers *et al.*, 1984; Reimers *et al.*, 1990; Hennessy *et al.*, 1997). Furthermore, in stress situations females showed higher C concentrations than males, as well as juvenile puppies seemed to have lower C levels than older subjects (Garnier *et al.*, 1990; Hennessy *et al.*, 1998). To date, to the author knowledge, only one study analysed C profile in canine neonates plasma, to investigate whether maternal corticosteroid treatment can improve neonatal vitality and alter maternal and neonatal endogenous C concentrations (Vannucchi *et al.*, 2012).

Recently, in adult dogs, hair C measurement was used for the diagnosis of hypercortisolism (Corradini *et al.*, 2013; Ouschan *et al.*, 2013), whereas very few studies were performed on hair C levels (Accorsi *et al.*, 2008; Finkler and Terkel, 2010; Galuppi *et al.*, 2013).

Although hair represents a promising matrix for hormones measurement in newborns, in fetus it starts to grow in late pregnancy (at about 45 days of gestation in dog), so that its analysis provides information limited to the last period of intrauterine development.

Another valuable matrix, providing cumulative cortisol exposure, is nails (Warnock *et al.*, 2010; Maidana *et al.*, 2013), collected without invasiveness and stored at room temperature. The nails start growing around the eighth week of pregnancy in humans, whereas by 40 days of gestation they are already formed in dogs (Pretzer, 2008), so that the cumulative information cover a longer window of time in comparison to the hair (Palmeri *et al.*, 2000; de Berker *et al.*, 2007; Ben Khelil *et al.*, 2011). Similarly to the hair, the hormones passively diffuse from

blood to the matrix of nails and are incorporated into the keratin along nail growth (Ben Khelil *et al.*, 2011). Recently, C was assessed in fingernails of adults humans beings (Warnock *et al.*, 2010; Ben Khelil *et al.*, 2011). In humans, fetal HPA axis starts to work as early as 8 weeks gestation (Mesiano and Jaffe, 1997), thus this hormone is available for incorporation into the nail throughout large part of pregnancy. The nails growth rate was investigated and it was reported variable between 1.9 and 4.4 mm/month, with a reasonable guide of 3 mm/month, or 0.1 mm/day (de Berker *et al.*, 2007). Under physiologic conditions, several maternal and fetal factors could influence C nails accumulation in fetus and newborn. Among maternal factors, the breed, age, and parity should be considered, even though Tegethoff *et al.* (2011) did not report an effect of these parameters on the concentrations of other steroids in infants nails. The neonatal main potential influencing factors are the age at the time of collection and gender. Since the hormones chronic incorporation, the analysis of nails collected at birth provides a retrospective picture of the hormones accumulation between the time of nails appearance to the time of birth. Subsequent timed nails sampling would provide information about the hormones incorporation in the interval between the two collections, as previously reported for hair C concentrations in newborn foals (Comin *et al.*, 2012). Concerning the fetal gender, several researches documented sex differences in some components of the HPA stress system, demonstrating the sex-specificity of many central and peripheric signals (Goel *et al.*, 2014). In human infants nails, Tegethoff *et al.* (2011) did not evidence differences between male and female newborns in some steroids levels.

Johnson *et al.* (2004), ten years ago, assessed cortisol concentrations in equine hoof to clarify the role of glucocorticoids in the pathogenesis of laminitis. Indeed, this hormone resulted detectable in the hoof, that could be maybe employed also in the newborn foals to perform retrospective hormonal evaluations. Recently, the C assessment in nails was validated even in bovine species, in which the claws, beyond the hair, can thus be used to provide retrospective hormonal information (Comin *et al.*, 2014). However, further studies are required to validate this technique in other animal species.

In the purpose to improve knowledge about fetal final stage of development and neonatal biology in canine species, the present study was aimed to assess the detectability of C in newborn puppies, by using new, non invasive matrices, such

as hair and nails, evaluating possible differences in C levels according to the newborn gender, breed size, and age, and, relatively to the hair, detecting the possible effect of the coat colour on the hormonal concentrations. Furthermore, the final aim was to evaluate possible correlations in C levels between the two biological matrices.

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CHAPTER 13

Hair and nails as new, non-invasive matrices for long time-frame cortisol analysis in newborn dogs

13. Hair and nails as new, non-invasive matrices for long time-frame cortisol analysis in newborn dogs

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13.1 Abstract

The last intrauterine fetal stage of development and the neonatal period represent the most challenging phases for the mammals offspring. In the dog, the knowledge about the final intrauterine fetal development and biology, as well as about the neonatal physiology, remains still scarce. Hormonal changes occurring in the last intrauterine fetal phase and during the early neonatal age are still not completely clear, probably because of the invasiveness related to the collection of the more common biological matrix, represented by circulating blood.

Toward the end of gestation, during parturition and after birth, the HPA axis is a key system regulating several physiologic processes, and its activity was investigated for a long time by blood analysis, considered an invasive and single point measurement. In respect to animal welfare and for a more correct long time-frame retrospective investigation, non-invasive hormonal studies were performed firstly in the hair of both humans and animals and, more recently, in the nails of human beings. This study was aimed to assess C in hair and nails of newborn puppies, and to evaluate the possible influence of the newborn gender, breed body size, and age on hair and nails C concentrations. The results obtained from 165 newborn puppies evidenced that hair and nails C levels were highly correlated each other ($P<0.0001$), although the C accumulation in the two matrices was different in relation to the class of age. Moreover, the puppies age

influenced both hair and nails C concentrations ($P<0.05$), with premature newborns showing higher values when compared to term born-dead puppies or puppies dead between 1 and 30 days of age. The present study demonstrated that C is quantifiable in hair and nails of newborn dogs, so that both matrices appear as interesting tools for new, non-invasive, long time-frame perinatal and neonatal researches also in canine species.

13.2 Introduction

The last intrauterine fetal stage of development and the neonatal period represent the most challenging phases for the mammals offspring. Contrary to other domestic species, in the dog the knowledge about the final intrauterine fetal development and biology, as well as about the neonatal physiology, remains still scarce. Hormonal changes occurring in the last intrauterine fetal phase and during the early neonatal period are still not completely clarified, probably because of the invasiveness related to the collection of the more common biological matrix, represented by circulating blood.

Toward term of pregnancy, the fetal hypothalamic-pituitary-adrenal axis (HPA) becomes a key system regulating several physiologic processes, such as the fetal multi-organs final maturation [1], the response to stress [2], and the triggering of parturition [3]. After birth and along the neonatal period, when the newborn undergoes a series of physiologic and metabolic changes necessary for survival and health, the HPA system still keeps a primary role in the neonatal adaptation to the extrauterine life [4, 5].

Activation of the HPA axis results in a cascade of endocrine responses. Cortisol (C), the main hormone of this axis, represents an appropriate biological endpoint in the investigation of HPA axis function. In response to the adrenocorticotrophic hormone (ACTH), the adrenals produce C, considered the best biomarker of chronic stress both for humans and animals [6]. In several animal species, HPA activity in the fetus, as well as in the newborn, was in the past investigated mainly by fetal blood [7] or cord blood analysis [8], but these methods were limited due to the invasiveness of sampling and to the single point measurement [2]. These limitations arose the need to detect alternative, non-invasive techniques for biological matrices collection, useful for the study of long time-frame hormonal changes in respect to both humans and animals welfare.

Alternatively to blood analysis, C levels were therefore assessed in the saliva, urine, and faeces, collected without invasiveness. Nevertheless, the saliva still remains representative of a single point hormonal measurement, whereas the urine and faeces assess the HPA activity over a period of only 24 hours or less. Recently, some studies reported C measurements in the hair of humans [9-13] and animals [14-19], providing the evidence that the hair could be considered a useful biological matrix for the non-invasive and long time-frame retrospective investigation of previous hormone accumulation. Interesting findings were reported about the effect of the pregnant condition on hair C accumulation. Particularly, D'Anna-Hernandez *et al.* found higher level of C in the hair of women in late pregnancy compared with those in early gestation or in the post-partum period, stressing the role of C in human late pregnancy [13].

Thanks to the lack of invasiveness at collection and the long time-window hormonal accumulation, the hair was proved to be an interesting tool for the study of C accumulation even in newborn babies. Indeed, hair C measurement was used to assess the effect of hospitalization in preterm neonates, in comparison with healthy ones [20]. The study highlighted that hospitalized newborns showed higher hair C levels compared with healthy babies. More recently, Comin *et al.* collected the hair of newborn horse foals to investigate the changes in C concentrations from birth to 60 days of age [18]. The authors reported the decline of hair C levels from birth to 30 and 60 days of age, probably as a response of the HPA axis during the neonatal adaptation to the extrauterine environment. Although hair C concentration was previously investigated in adult dogs [15, 21], the authors are not aware of similar studies in the newborn puppy.

In humans, another valuable matrix providing a long-term view of C accumulation is fingernail [22-24], collected without invasiveness and easily stored at room temperature. Similarly to the hair, also fingernails analysis allows the assessment of hormones accumulated over a long-time period, passively diffused from capillaries to the matrix of nails, and incorporated into the keratin along nail growth [24]. To the authors knowledge, to date similar studies were not performed neither in newborn babies nor in animals. Both the hair and nails, thanks to lack of invasiveness at collection and because of the long time-frame retrospective accumulation, could therefore represent interesting biological

matrices for the study of C accumulation during the fetal and neonatal periods also in canine species.

The possible effect of some parameters, such as the ethnicity or race, gender, age, and coat colour on hair C accumulation was widely investigated in humans and animals, providing conflicting results [6].

In the purpose to improve knowledge about the final stage of fetal development and neonatal biology in dogs, by using non-invasive methods, the aims of the present study were: 1) to assess the detectability of C in newborn puppies, through new, non-invasive matrices, such as hair and nails; 2) to evaluate possible influence of the newborn gender, breed size, and age on C concentrations, in both hair and nails; 3) relatively to the hair, to detect also the possible effect of coat colour on C levels; 4) to evaluate possible correlations and differences between the C concentrations in the two matrices.

13.3 Materials and methods

13.3.1 Animals

The study was performed on 165 spontaneously dead purebred puppies, 80 females and 85 males, belonging to several canine breeds, without gross physical malformations. All the owners and breeders gave a written informed consent to the collection of hair and nails from dead puppies, for research purposes.

According to maternal body weight, the 165 subjects were classified as belonging to small size breed (bodyweight ≤10 kg) (N = 60), or to a merged class of medium-large size breed (bodyweight >10 kg) (N = 105). Based on the age at death, all newborns were grouped in three classes of age, as follows: 1) premature: when puppies were delivered between 2 and 1 weeks before the expected date of whelping, estimated by ultrasonographic parturition date prediction [25] (N=25); 2) fresh, term-born-dead puppies (puppies delivered dead at the predicted term of pregnancy and puppies born alive at term, but dead within 24 hours since birth (N=97); 3) puppies dead between 1 and 30 days of age (N=43).

13.3.2 Hair and nails samples collection

A proper amount of hair (at least 20 mg) was carefully shaved from each puppy. Hair was collected closest to the skin always from the same area [26], including only the back and the dorsal portion of the neck. Given the conflicting results reported in bibliography concerning the influence of coat colour on C concentrations, when in the same puppy both light and dark hair areas were detected in a sufficiently large surface to allow the collection, both dark and light hair were separately collected. The nails of all the fingers were trimmed and pooled (at least 4 mg). Hair and nails samples were separately stored in dry tubes, at room temperature, until analysis.

13.3.3 Hair and nails cortisol assays

Hair strands and nails were washed in 5 ml isopropanol to minimize the risk of extracting C from outside these biological samples and to ensure the removal of any steroids on their surface. Hair and nails C was extracted with methanol and measured by RIA [27]. All the samples were freeze-dried as described by Comin *et al.* [27] and the dry weights were calculated.

13.3.4 Statistical analysis

Data regarding C levels in hair and nails were statistically analysed by ANOVA, in order to assess: a) possible differences in hair or nails C concentrations according to the newborn gender and to breed size; b) possible differences in hair or nails C levels according to the newborn class of age; c) possible differences in hair C concentrations according to the coat colour. Furthermore, the Tukey test was used to define the possible differences in hair and nails C levels among the three classes of age. Differences between C concentrations in the two matrices within each class of age were assessed by t-test corrected for unequal variances. Finally, possible correlations between hair and nails C levels were analysed by Spearman correlation test. Significance was set at $P<0.05$.

13.4 Results

Cortisol was detectable in all the hair and nails samples belonging to the 165 newborn puppies. Specifically, in the hair of these subjects, the overall C concentration (mean ± SD) was 65.2 ± 52.23 pg/mg, whereas in the 165 pooled samples of nails, the overall C concentration was 62.6 ± 58.8 pg/mg. The

Spearman correlation test revealed a highly significant positive correlation between hair and nails C levels ($R=0.68$, $P<0.0001$).

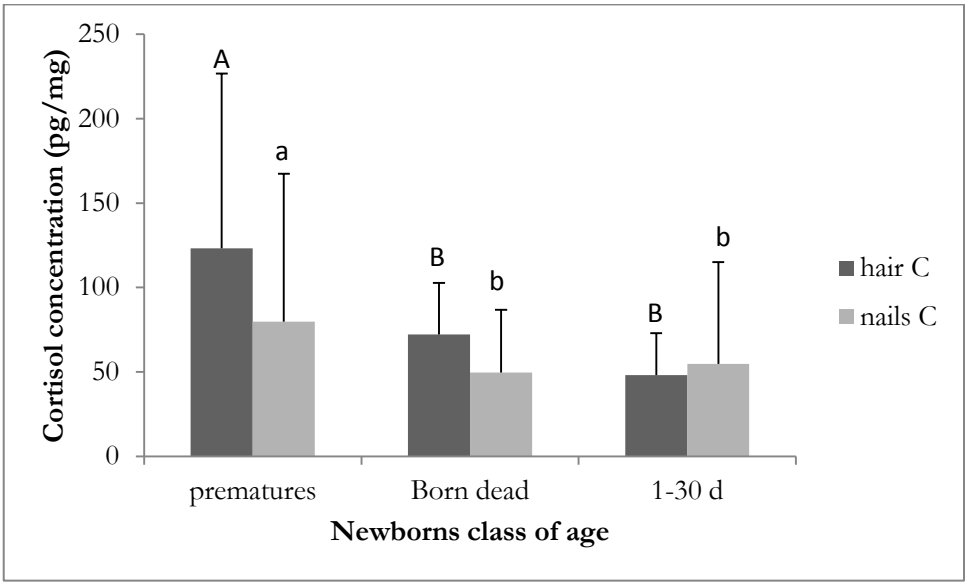
The statistical analysis did not show any difference in C concentrations neither in the hair nor in the nails in relation to the newborn gender or breed body size (Table 1), whereas a significant effect of the class of age was found on both hair and nails C levels, with significant higher values in premature compared with born dead puppies or puppies dead between 1 and 30 days of age (Figure 1). From the 165 newborns, based on the coat colour, 68 light and 108 dark hair samples were collected and analysed for C concentrations. The results showed a mean (± SD) of 67.6 ± 55.81 pg/mg in light hair compared to 63.0 ± 50.06 pg/mg in dark hair, without significant differences.

When the possible differences in C levels between the two matrices within each class of age were assessed, the statistical analysis documented the absence of differences between the C concentrations in the two matrices within the class of premature puppies (mean ± SD: 123.22 ± 103.41 vs. 79.81 ± 87.49 pg/mg, in the hair and nails, respectively), whereas in term born-dead subjects C levels were higher ($P<0.05$) in the hair compared to the nails (mean ± SD: 66.97 ± 36.56 vs. 53.92 ± 49.91 pg/mg, in the hair and nails, respectively). On the contrary, in the class of age ranging between 1 and 30 days, C concentrations were significantly higher ($P<0.0001$) in the nails compared to the hair (mean ± SD: 42.97 ± 18.84 vs 77.37 ± 55.29 pg/mg, in the hair and nails, respectively).

Table 1: Hair and nails C concentrations in the 165 puppies, according to newborn gender and breed size (mean ± SD).

	Newborn gender		**Breed size**	
	Females (N=80)	**Males (N=85)**	**Small (N=60)**	**Medium-large (N=105)**
Hair C (pg/mg)	57.4 ± 43.22	71.9 ± 59.03	57.9 ± 45.85	84.6 ± 61.77
Nails C (pg/mg)	60.1 ± 60.76	65.1 ± 57.12	59.0 ± 57.74	64.6 ± 59.52

Figure 1: Concentrations of C in hair and nails of the 165 puppies, grouped according to the class of age (mean ± SD).



a, b and A, B $P<0.05$

13.5 Discussion

The present study showed for the first time that C is detectable in hair and nails of newborn dogs, demonstrating the usefulness of both hair and nails as non-invasive, alternative matrices for perinatal hormonal investigations, also in canine species.

Because the body hair and color marking in dogs appear 44-46 days post coitum [28], it is reasonable to assume that the collection of hair at birth should reflect the hormones accumulation from the time of hair appearance to the time of puppies delivery. Thus, in canine species, the C concentrations measured in hair collected at birth would depict the hormone accumulated during the last two-three weeks of pregnancy. To the authors knowledge, specific studies about the proportion of maternal C crossing the placenta and activating the fetal HPA axis are lacking in the dog. Nevertheless, some researches in humans and other animal species demonstrated that maternal C activates the fetal pituitary-adrenal axis [29-31], so that C accumulated in the fetal hair during gestation could be produced by the mother, by the fetus itself, or by both mother and fetus.

The authors are not aware of references about the exact timing of nails first appearance in canine fetus but, at 30 days of gestation, full visible nails are usually detectable (authors observation). This means that C measured in nails collected at birth would reflect a longer time of accumulation when compared to the hair, whereas the question about the exact maternal, fetal, or cumulative origin of the hormone still remains unsolved.

The overall mean concentrations of C were similar in hair and nails (65 vs. 63 pg/mg), and C levels in these two biological samples were highly correlated, suggesting that both matrices are representative of the HPA activity in the newborn puppy. Thus, even the nails could work as an interesting tool for non-invasive, long time-frame fetal and neonatal studies in canine species. In addition, nails seem to be more useful compared to the hair for two main reasons. Firstly because the amount of hair necessary for the analysis is relevant (about 20 mg) as regard to the small size of newborn puppies, so that a large surface of the body should be shaved. This was not a problem for the current study because all the subjects enrolled were dead, but the future application of this procedure to viable newborn puppies could be a problem. The nails, instead, require a smaller amount of sample to enable the hormone measurement. Secondly, the earlier appearance of the nails during the fetal development allows the analysis of C accumulated during a longer time in comparison to the hair.

When the possible influence of the newborn gender or breed body size on hair and nails C concentrations was assessed, no significant effect was found, most likely because of the wide standard deviations, indicating high inter-individual variations. Slightly higher mean C levels were detected in the hair of males compared to females puppies, nevertheless the difference between the two genders appeared not significant, in agreement with the absence of sex influence on hair C concentrations reported by Comin *et al.* in newborn horse foals [18]. Literature from humans provided conflicting results about this topic. In fact, while some authors reported the absence of a confounding effect of sex on hair C concentrations [9, 32-34], Dettenborn *et al.* suggested higher values in males when compared to females, in adult humans [35].

The lack of breed body size effect on hair C concentrations seems to be in contrast with previous findings reported by Fraser *et al.* [36], who showed a

positive correlation between body mass index and urinary C levels in humans. The different biological matrix used could be the reason for different results.

The statistical analysis failed to find any significant difference in C concentrations between light and dark hair. Both in humans and animals, the conflicting results about the effect of hair colour on C levels were fully debated in the review by Russell *et al.* [6], so that the lack of a significant effect in newborn puppies is not surprising.

Concerning the nails, the possible effect of the gender, body mass index, or age on C concentrations was still not investigated in the few studies performed in humans, although the effect of gender on adult human nails growth [37] and possible C accumulation [22] was reported.

The most relevant result was the high significant effect of the class of age on C levels in the hair, as well as in the nails, with significantly higher values in puppies delivered prematurely when compared to puppies dead at birth or within the first 30 days of age. A previous investigation in human infants [20] highlighted higher hair C concentrations in hospitalized preterm born infants compared to healthy, born at term, neonates. Since the hospitalized infants were born at younger gestational age, in comparison to the healthy newborns, the effect of age could create a potential confusion [6]. On the other hand, it is well known that in women, in the third trimester of pregnancy, hair C levels appear higher than those during the first trimester or the post-partum period [13]. Because in the present study it was not possible to ascertain the maternal or fetal (or both) origin of C accumulated in the hair and nails of premature puppies, it is reasonable to suppose that the higher C levels observed in both matrices belonging to premature puppies could be the result of maternal increased C circulating concentrations, as reported for late pregnant women. Unfortunately, in the present study hair was not collected from pregnant bitches, so that this hypothesis still remains to be proved. In humans, it was suggested that even early detection of stress stimuli by the placenta induces the release of CRH with consequent increased risk for preterm birth [38]. It is however known that the effects of glucocorticoids in primates parturition is rather different from other animals species [39].

Interestingly, also higher nails C levels were detected in premature compared to born dead subjects and puppies dead within the first 30 days of age. The only

one study investigating this hormone in human nails was performed on students aged between 18 and 24 years, and the effect of age was not assessed [22].

Decreasing hair C concentrations from birth to 60 days of age were reported in newborn horse foals by Comin *et al.* [18]. At birth, hair C median levels in horse foals were about 55 pg/mg, at 30 days about 30 pg/mg, and at 60 days of age about 16 pg/mg. In the present study, hair C mean concentrations in term-born dead puppies were about 72 pg/mg, and between 1 and 30 days of age about 48 pg/mg, a bit higher than in newborn horse foals. Unfortunately, unlike the horse foals, hair C concentrations measured in puppies aged 1 to 30 days enrolled a more heterogeneous age population, explaining, at least partially, the wide inter-individual variation. In addition, also the cause of death could have influenced the C accumulation within this class of age. On the other hand, a wide inter-individual variation in hair C concentrations was observed in all the three classes of age, even when the puppies gender or the breed body size were concerned. This finding highlights that many are the parameters affecting the C accumulation in the hair of canine fetuses and newborns. Comparing the mean hair C level measured in puppies of all the three classes of age with data reported by Accorsi *et al.* [15] in adult dogs, the hair C mean concentration in newborn puppies was definitely higher than in adults (about 123 pg/mg in premature puppies, about 72 pg/mg in term born-dead puppies, and 48 pg/mg in 1-30 days old puppies vs. 2.1 pg/mg in adult dogs, respectively).

It is interesting to note that, also in the nails, the class of age affected significantly the C levels. Unfortunately, the authors are not aware of studies investigating the effect of age in nails C concentrations neither in humans nor in other animals. However, in an elegant review about nail biology [40], it was suggested that nails growth is affected by several factors, among which the age of the subject, with children under 14 having faster growth than adults.

It was finally surprising the finding of the reverse C accumulation in the hair and nails between term born-dead puppies and puppies 1 to 30 days old, suggesting a different pattern of C incorporation in the two matrices in the perinatal period of dogs that deserves further investigations.

13.6 Conclusions

This study, for the first time, demonstrated that C is quantifiable in hair and nails of newborn dogs, so that both matrices are interesting for non invasive, long time-frame fetal and neonatal studies also in canine species. The most relevant finding was the significant effect of the class of age on both hair and nails C concentrations, with higher values in puppies delivered prematurely compared to term-born dead puppies or puppies dead within the first 30 days of age. Newborn gender, breed body size and, relatively to the hair, the coat colour do not seem to affect hair or nails C accumulation during the foetal development or the first 30 days after birth. The maternal or fetal origin (or both) of this hormone, as well as the influence of gender, breed size, or coat colour deserve further investigations in normal puppies, but also open new perspectives for the study of all the maternal and fetal factors that could affect fetal and/or neonatal development and well-being. Finally, also the different pattern of C accumulation between the hair and nails within different classes of age appears an interesting topic to investigate much deeper.

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CHAPTER 14

Bacterial infections in the newborn

14.1 Neonatal bacterial infections in humans and large animals

Neonatal bacterial infections continue to be a significant health care burden in both humans and domestic animal species.

In humans, neonatal sepsis represents a common problem especially in infants with a very low birth weight. Group B streptococcal infection is still considered the major cause of neonatal bacteremia, although the intra-partum antibiotic prophylaxis strongly decreased its incidence. However, some researches performed on preterm neonates documented also *Escherichia coli* (*E. coli*) as a common responsible for the early-onset sepsis. In fact, this bacterium was recognized as the major pathogen of neonatal bacteremia in premature infants and the second cause in term babies. It was documented that Gram-positive organisms are mainly involved in late-onset sepsis, with coagulase-negative staphylococci responsible for 48% of infections (Shah and Padbury, 2014).

In calves, the bacterial enteritis remains to date the most frequent neonatal disease and *E. coli* appears the first bacterial organism involved, above all the enterohaemorragic strains within the first week of age. This enteritis can leads to the colisepticaemia, more commonly within the first 3 days of age and with a high mortality rate (Butler and Clarke, 1994; Taverne, 2013). Respiratory diseases represent the second most important cause of morbidity and mortality in calves, nevertheless the information about their incidence is scarce, probably due to the breeder lack of interest. In bovine species, the respiratory diseases have a multifactorial etiology, being usually caused by viral infections, that predispose successively to bacterial complications. At this regard, *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* were reported to be the microbial organisms likely involved (Taverne, 2013; Jamali *et al.*, 2014; Taylor *et al.*, 2014; Francoz *et al.*, 2015). Interestingly, *Histophilus somni* was judged able to cause fatal septicaemia in 10% of cases, with neurologic and poliarthritic clinical signs (Taverne, 2013).

Several studies performed on equine species documented the bacterial infection as a leading cause of death in foals during the first few weeks of age (Henson and Barton, 2001; Marsh and Palmer, 2001; Corley *et al.*, 2007; Theelen *et al.*, 2014). Both Gram-negative and Gram-positive bacteria were reported to be involved: specifically, among the first, *E. coli*, *Pasteurella* spp., *Salmonella* spp., *Klebsiella* spp., *Actinobacillus* spp., *Enterobacter*, and *Pseudomonas* spp. are included, whereas, among the second, β-haemolytic streptococci, stafilococci spp., and

Corynebacterium are recognized (Castagnetti, 2013). However, *E. coli* and other Gram-negative organisms remain the most common isolates from septicaemic neonatal foals, although the Gram-positive prevalence is increasing (Marsh and Palmer, 2001; Theelen *et al.*, 2014). The gastrointestinal tract is considered the principle portal of entry for bacteria (Henson and Barton, 2001; Marsh and Palmer, 2001; Corley *et al.*, 2007). In fact, the strong association between bacteremia and enteritis, as well as with pneumonia and omphalitis, was evidenced in equine newborn (Magdesian, 2005; Hollis *et al.*, 2008; Castagnetti, 2013).
The incidence of septicaemia was investigated also in piglets (Bressack *et al.*, 1987; Graage *et al.*, 2014). In this species, group B streptococci and *Staphylococcus aureus* were documented as the major responsible for this pathology.

14.2 Bacterial infections in canine newborn

Neonatal mortality rate in puppies was reported to range from 9-10% to 26-34%, subsequent to complicated and uncomplicated whelpings, with a greatest risk during the first week of age (Mosier, 1981; Davidson, 2003; Peterson, 2011). The most neonatal deaths occur due to any kind of dystocia (Münnich *et al.*, 1996; Moon *et al.*, 2000; Moon-Massat and Erb, 2002); in this regard, differences in the number of stillbirths were not observed according to the puppies gender and their frequency seemed to be not dependent on the number of newborns for litter, contrary to post-partum mortality, that is more common in large litters. A recent study evidenced, however, that perinatal mortality is significantly influenced by breed, litter number and size, and bitch age (Tønnessen *et al.*, 2012). The second cause of neonatal morbidity and mortality in canine species is represented by bacterial infections, which can be remain localized or become generalized leading to septicaemia, the most frequent cause of mortality within the first 14 days of age (Sager and Remmers, 1990; Poffenbargerm *et al.*, 1991; Münnich *et al.*, 1995; Van der Beek *et al.*, 1999; Davidson, 2003; Daniels and Spencer, 2011).

14.2.1 Septicaemia predisposing factors

In the predisposition to septicaemia several factors should be considered, such as the bitch, the process of whelping, the environment, the newborn itself, and the

overcrowding in the kennel. Maternal predisposing factors include uterine and transplacental infections, high bacterial contamination in the parturition canal, puerperal endometritis and mastitis and/or other diseases (gastroenteritis, periodontitis, dermatitis), unhealthy diet along pregnancy or nursing, lactation diseases (agalactia) or poor quality of colostrum, whereas dystocia, prolonged expulsive phase or mishandling midwives represent possible risk factors during delivery. At birth, unsuitable temperature, overcrowding, and/or poor cleaning of breeding may adversely affect on healthy status of the newborn puppies. The infection could spread from mother to fetus during gestation, delivery or, after whelping, throught infected maternal secretions, as vaginal and oronasal discharges, faeces, and milk (Sager and Remmers, 1990; Münnich *et al.*, 1996; Münnich and Lübke-Becker, 2004). Also some specific conditions of the newborn itself can predispose to septicaemia: low birth weight (Indrebø, 2007), prematurity, congenital abnormalities, hypoxia, hypothermia, hypoglycemia, localized infections (omphalitis, gastroenteritis, pneumonia, dermatitis), and inadequate intake of colostrum, as well as the spread use of some antibiotics (ampicillin), and inbreeding (Sager and Remmers, 1990; Poffenbargerm *et al.*, 1991; Go *et al.*, 1994; Münnich *et al.*, 1995; Van der Beek *et al.*, 1999; Johnston *et al.*, 2001; Davidson, 2003; Peterson *et al.*, 2011; Philbey *et al.*, 2013). Therefore, bacterial colonization is allowed and defensive capabilities of newborn, still not completely efficient, are ineffective. Even bacterial traslocation, that is the passage of live bacteria through intact intestinal barrier, is recognized as a cause of systemic disease, above all in newborns (Go *et al.*, 1994; Dahlinger, 1997).

14.2.2 Bacterial aetiology and clinical course

Among bacteria, the organisms most frequently associated with neonatal mortality in dogs are *E. coli*, staphylococci (above all *Staphylococcus aureus*), streptococci (in particular *Streptococcus canis*), and klebsielle (especially *K. pneumoniae*) (Askaa *et al.*, 1978; Mosier, 1981; Sager and Remmers, 1990; Johnston *et al.*, 2001; Davidson, 2003; Daniels and Spencer, 2011).
E. coli is a normal saprophytic bacterium of enteric flora but some strains can induce, with different mechanisms of action, enteritis and always fatal septicaemia. The newborn puppy enteric epithelium is more permeable to *E. Coli* than adult dogs (Young *et al.*, 1983). The main infection sources are represented by mother (Bjurström and Linde-Forsberg, 1992; Münnich and Lübke-Becker,

2004), or other subjects living in the kennel, and environment (Allen and Dagnall, 1982; Münnich, 2008). *E. Coli* was the most frequently isolated bacterial species from the vagina of bitches with neonatal mortality, as reported by Bjurström (1993) and subsequently confirmed by Münnich and Lübke-Becker (2004), which showed that 60% of *E. Coli* strains isolated from dead puppies were also found in vaginal samples of the dams, demonstrating their maternal origin. However, *E. Coli* can be normally isolated in bitch vagina (Bjurström and Linde-Forsberg, 1992). The role of canine milk is still discussed because, in the most cases of neonatal septicaemia, bacteria isolated from canine milk do not seem to be the primary cause of neonatal mortality (Schäfer-Somi *et al.*, 2003; Münnich and Lübke-Becker, 2004).

Among staphylococci, the principal pathogenic species are *S. aureus* e *S. intermedius*, the first responsable for pyodermitis and the second for localized infections (otitis, mastitis, pyometra). *S. aureus* was described as a frequent cause of neonatal septicaemia (Sager and Remmers, 1990; Münnich *et al.*, 1995), whereas *S. intermedius* is unlikely to be a major cause of septicaemia in newborns. The process of neonatal colonization by *S. intermedius* in puppies is still discussed: it seems to happen soon after birth (Saijonmaa-Koulumies and Lloyd, 2002) or gradually during the first weeks of age (Allaker *et al.*, 1992), with a speed proportional to the extent of environmental and/or maternal contamination. Genetic relationship between bacterial strains in puppies and their mothers was documented for staphylococci as well as for *E. Coli* (Münnich, 2008).

Streptococci are opportunistic pathogens that normally reside in upper respiratory, intestinal, lower urinary and genital tracts (Bjurström and Linde-Forsberg, 1992) and could be responsible for localized infections (overall dermatitis and pneumonia) or septicaemia in dogs of all ages (Lamm *et al.*, 2010). *β-hemolytic streptococci* are the most pathogen streptococci for the animals and the more frequently isolated strains in canine species are *S. canis*, *S. dysgalactiae* ssp *equisimilis*, and *S. equi* ssp *zooepidemicus* (Vela *et al.*, 2006; Lamm *et al.*, 2010). Streptococcal septicaemia is one of the most common cause of miscarriage or neonatal death in the dog (Kornblatt *et al.*, 1982; Kornblatt *et al.*, 1983; Bjurström, 1993): puppies can become infected during the intrauterine life or at the time of birth with the passage through vagina (Vela *et al.*, 2006). Indeed, streptococci often were isolated from bitches vaginal discharges (Greene and Prescott , 1998). Consumption of contamined milk represents another possible source of infection, but at a less extent (Schafer-Somi *et al.*, 2003).

Klebsielle normally reside in the intestinal tract and the most important species is *K. pneumoniae*. As concerns at *K. pneumonia*, the use of antibiotics that reduce resistance to colonization, nutrition with commercial formulas, and nosocomial transmission seem to predispose the newborn puppy to the infection.
Also *Proteus mirabilis* and *Pseudomonas aeruginosa* were isolated in cases of neonatal death. *P. mirabilis*, largely widespread in nature and often in faeces, overall in dogs, can induce diarrhea in young subjects. *P. aeruginosa*, instead, is a bacterium frequently involved in diseases primarily caused by other bacteria; immunosuppressive or too long antibiotic therapies are predisposing factors for the infection. *P. aeruginosa* pathogenic activity often causes suppurative inflammation, localized or generalized, with a possible fatal exitus.
Hyperacute form of septicaemia is characterized by fast fatal exitus, with asymptomatic death of the newborn puppy (Askaa *et al.*, 1978; Davidson, 2003; Daniels and Spencer, 2011), whereas the subacute form is defined as "fading puppy syndrome". In this latter the newborns, apparently healthy and with a normal weight at birth, die within the earliest days of age (Johnston *et al.*, 2001; Indrebø, 2007).
The clinical management of infected newborn puppy is very difficult because of the fast onset of aspecific symptoms and disease course; thus, the prognosis is often poor. Clinical signs compatible with a possible septicaemia may not be noted because death often occurs suddenly or include a decrease in weight gain, loss of the sucking reflex, weakness, cyanosis, persistent wathery diarrhea, hematuria, unusual vocalizations, abdominal distension and/or pain, hypothermia, necrosis and sloughing of the extremities and coma (Johnston *et al.*, 2001; Davidson, 2003). In newborn puppy the knowledge about pharmacodynamics and pharmacokynetics is scarce. Third generation cephalosporins and fluorquinolones were reported previously as the most effective drugs in case of neonatal septicaemia (Johnston *et al.,* 2001; Davidson 2003; Münnich, 2008; Daniels and Spencer, 2011; Veronesi, 2013). Nevertheless, unfortunately the bacterial antimicrobial resistance and multiresistance are emerging, probably due to the spread use of the antibiotics (Guardabassi *et al.*, 2004; Milani *et al.*, 2012).

14.2.3 Diagnosis

Post-mortem examination of the newborn puppy, including necropsy, bacteriology, and histological analysis (Farstad, 2003; Jonhson, 2006; Morris *et al.*, 2007; Veronesi, 2013), could be helpful to confirm the suspected diagnosis of neonatal septicaemia and to identify the possible cause of death. The isolation of the involved bacterial strains would allow to perform the antimicrobial susceptibility test, for choosing the most rationale therapy in surviving littermates as well as for better managing of the further pregnancies in bitches with a previous history of neonatal losses (Veronesi, 2013).

Despite the high rate of neonatal bacterial infections evidenced in canine species, to date the literature provides only scarce information about this interesting topic. Thus, the present study was born to investigate the real bacterial aetiology of the neonatal mortality in dogs and to evaluate the *in vitro* antibiotic sensitivity of the isolated bacterial strains.

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CHAPTER 15

A survey on bacterial involvement in neonatal mortality in dogs

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15. A survey on bacterial involvement in neonatal mortality in dogs

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15.1 Abstract

Bacterial infections represent the second cause of neonatal morbidity and mortality in dogs, so the present study aimed to investigate the bacterial involvement in canine neonatal mortality and to evaluate the antibiotic susceptibility of the isolated bacteria. Fifty-one newborn purebred puppies, born dead or dead within 28 days of age, belonging to 36 different litters, were enrolled and the following procedures were performed on their fresh dead bodies: necropsy, collection of swabs by liver, kidney, lung, small bowel, and possible thoracic and/or abdominal effusion, for both bacteriological examination and antimicrobial susceptibility testing, and collection of samples by the same organs for histology. About 47% of total swabs were positive at bacteriology (pure bacterial culture or bacterial association). In 65% of the newborns puppies the mortality could be attributed to a bacterial infection. Although the high multidrug resistance, the most effective antimicrobials were third generation cephalosporins and fluoroquinolones. In case of neonatal

mortality, bacterial culture and antimicrobial susceptibility testing become essential for a targeted therapy in surviving littermates and for the management of following pregnancies in bitches with recurrent neonatal loss.

15.2 Introduction

Neonatal mortality rate in canine species ranges from 9 to 34% (8, 15, 25, 37), with a greatest risk during the first week of age (8, 25, 26, 30). Beyond dystocia (22, 23, 28), bacterial infection was identified as the second main cause of neonatal death in dogs. Indeed, the consequent septicaemia is thought to be the most common responsible for puppies mortality within the first 21 days of age (7, 27, 35, 37). Bacterial infection often spreads from mother to foetus during pregnancy, delivery or, after whelping, through infected maternal secretions, in particular vaginal and oronasal discharges, faeces, and milk (29, 33). Bacterial translocation is also recognized as a cause of neonatal systemic disease (6, 10).

The microbial organisms most frequently associated with neonatal death are *Escherichia coli* (1, 13), *Staphylococcus aureus* and *Staphylococcus pseudintermedius* (27, 32), *Streptococcus canis*, *Streptococcus dysgalactiae* subsp *equisimilis*, and *Streptococcus equi* subsp *zooepidemicus* (11, 19), and *Klebsiella pneumoniae* (8, 26). *Proteus mirabilis* and *Pseudomonas aeruginosa* were also isolated in cases of neonatal loss (26).

Septicaemia may have a hyperacute evolution, with sudden death of the neonates (1, 7, 8, 37), or a subacute course (14, 16, 37). The clinical management of septicaemic newborn puppy is very difficult because of the sudden onset of unspecific symptoms and the fast disease course. Thus, treatment is usually delayed and unsuccessful, and the prognosis is poor.

Post-mortem examination, including necropsy and additional investigations (15, 37), could be helpful to identify the possible cause of neonatal mortality. The detection of involved bacteria allows for performing the subsequent antimicrobial susceptibility testing (AST), essential to choose the most appropriate therapy of surviving littermates and for a better management of following pregnancies in the same bitch.

For all these reasons, the aims of the present study were the investigation of the bacterial involvement in puppies neonatal mortality and the evaluation of the antibiotic susceptibility of the isolated bacteria.

15.3 Materials and methods

15.3.1 Animals

The study was performed in Northern Italy, between January 2012 and May 2013, on 51 full term newborn puppies, belonging to 36 litters of 17 breeds. All these puppies were stillborn or dead during the neonatal period, considered as the first 28 days of age (8, 37). The 36 bitches, 2-8 years old, 20 primiparous and 16 pluriparous, were healthy before mating, regularly submitted to a vaccination program, and correctly dewormed. Among these, 7 female dogs revealed previous isolated or recurrent, not investigated, neonatal losses. In all the bitches the last gestation showed a normal clinical course.

15.3.2 Necropsy and sampling

Only fresh dead puppies (stored at 4°C for an elapsing time from death to necropsy of maximum 4 hours) underwent the necropsy, that was mainly focused to a correct bacteriological investigation. For each newborn, body size, maturity, sex, and weight were recorded; gross malformations, when present, were detected. After the careful opening of the abdominal cavity, swabs were collected from liver, kidney, and small bowel, avoiding any possible contamination, and from abdominal effusion, if present. Afterwards, the same organs were also sampled for histology and the specimens were immediately fixed in 10% buffered formalin solution. Finally, the thoracic cavity was opened; lung and possible thoracic effusion were sampled as reported for the abdomen. Only one specimen was collected for each organ.

15.3.3 Bacteriological examination and antimicrobial susceptibility testing

The swabs were immediately plated on Petri plates with first isolation medium (TSA with 5% sheep blood, Oxoid, Milan, Italy), by streaking technique, to obtain the growth of bacterial colonies. Plates were incubated at 37°C for 24 hours under aerobic conditions; swabs collected by lung and effusions were also incubated in modified atmosphere in a candle jar (5% CO_2). After the first incubation, all the plates that were bacteriological negative underwent a second

incubation at 37°C for 24 hours; the plates were considered sterile when bacterial colonies were not observed after the second incubation.
Isolated bacteria were identified by using different techniques: the macroscopic observation of colonies morphology on blood-agar plates, Gram-stain reaction, cellular morphology, and biochemical tests, particularly catalase and oxidase tests (Oxichrome Reagent, Remel-Oxoid, Milan, Italy). For the identification of Gram-negative bacteria (*Enterobacteriaceae*), the growth on selective and differential medium Mc Conkey (Oxoid, Milan, Italy) was evaluated. Moreover, commercially available specific miniaturized methods ("API-20E"®, "API-20NE"®, "API-20STAPH"®, Bio-Mériéux, Craponne, France), as well as selective and differential media, such as Mannitol Salt Agar (Oxoid, Milan, Italy) and Brilliant Green Agar (Oxoid, Milan, Italy), were carried out to achieve the biochemical characterization of bacteria (4).
For each cultured bacterial species, susceptibility to the most common antimicrobial drugs was investigated according to CLSI guidelines (5). Ampicillin, amoxicillin, amoxicillin and clavulanic acid, cephalexin, ceftriaxone, enrofloxacin, metronidazole, spiramycin, polymyxins, and trimethoprim-sulfamethoxazole were tested. All the bacteria were classified as being susceptible, intermediate, or resistant to antibiotics.

15.3.4 Histology

Samples were evaluated for possible inflammatory lesions, bacterial emboli within the blood vessels or internal organs, and bacterial growth within the deep tissues.
Four-micrometer-thick serial sections were obtained from each paraffin block and stained with hematoxylin-eosin (H&E).

15.4 Results

15.4.1 Clinical findings

Out of the 51 newborn purebred puppies (32 born by euthocic delivery and 19 by dystocic delivery), 9 were born dead and 42 died within 28 days after birth. Among the latter ones, 4 newborns died suddenly, 20 displayed noticeable symptoms within 48 hours from birth, whereas the other 18 showed clinical

signs after 2 days of age. In 26 of 36 litters, more than one newborn puppy was affected by the same symptoms. Clinical signs were always unspecific, sometimes associated, and distributed as follows: lethargy (54.9%), loss of suckling reflex (41.2%), abnormal vocalizations (21.6%), diarrhea (17.6%), failed weight gain (13.7%), hypothermia (5.9%), dermatitis (5.9%), conjunctivitis (5.9%), rigidity (5.9%), dyspnoea (2%), convulsions (2%), jaundice/regurgitation (2%), and heart murmur (2%). Clinical course was fast in 35 puppies, with death within 48 hours since the onset of clinical signs.

15.4.2 Necropsy

Necropsy evidenced that all the newborns were well developed, mature, and of the correct size for the belonging breed. Gross malformations were never detected. Thoracic effusion was found in 6 puppies, whereas abdominal effusion in 4 subjects. In most cases, gross organic lesions were not present, although areas of lung atelectasis were observed in 16 puppies.
A total of 214 swabs were collected. In all the 51 subjects the following organs were sampled both for bacteriology and histology: liver, kidney, lung, and small bowel. Swabs were taken also from thoracic effusion and abdominal effusion in 6 and 4 cases, respectively.

15.4.3 Bacteriology and antimicrobial susceptibility test

From a total of 214 swabs, 101 (47.2%) were bacteriological positive, whereas 113 (52.8%) were bacteriological negative. For 39 of 51 (76.5%) newborn puppies, at least one organ resulted positive at bacteriological examination. In 87 of 101 positive swabs (86.1%), the following bacteria were isolated in pure culture: *E. coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *haemolytic Escherichia coli*, *Proteus mirabilis*, *β-haemolytic streptococci*, *Klebsiella pneumoniae*, *Staphylococcus pseudintermedius*, *Bacillus*, and *Streptococcus faecalis*. Their distribution in the organic swabs is reported in Table I. In the other 14 postive samples (13.9%), different bacterial associations were found, as showed in Table II.

Table I - Organic distribution of bacterial strain isolated in pure culture in 87 samples collected from full term newborn puppies in Northern Italy, between January 2012 and May 2013.

Bacteria	Liver	Kidney	Lung	Small bowel	Abdominal effusion	Thoracic effusion	Total samples
E. coli	12	10	14	13	1	-	50
Haemolytic E. coli	-	2	1	2	-	-	5
S. aureus	1	2	2	1	-	1	7
β-haemolytic S.	-	-	3	-	-	-	3
K. pneumoniae	-	1	1	-	-	-	2
P. mirabilis	2	1	1	1	-	-	5
S. pseudintermedius	-	-	1	-	-	-	1
E. faecalis	1	2	1	6	-	-	10
Bacillus	-	-	-	1	-	-	1
S. faecalis	-	-	-	1	-	-	1
A. viridans	-	-	1	-	-	1	2[a]

[a] *A. viridans* was isolated in lung and thoracic effusion samples belonging to the same subject.

Table II - Organic distribution of bacterial association isolated in 14 samples, collected from full term newborn puppies in Northern Italy, between January 2012 and May 2013.

Bacterial association	**Liver**	**Kidney**	**Lung**	**Small bowel**	**Abdominal effusion**	**Thoracic effusion**	**Total samples**
E. coli + *K. pneumoniae*	2	2	-	2	1	-	7
E. coli + *E. faecalis*	1	-	-	1	-	-	2
Haemolytic E. coli + *E. faecalis*	-	-	1	1	-	-	2
P. mirabilis + *E. coli* + *P. aeruginosa*	-	-	-	1	-	-	1
P. mirabilis + *E. coli*	-	-	-	1	-	-	1
E. coli + *haemolytic E. coli*	-	-	-	1	-	-	1

On the basis of these results, it is reasonable to suppose that 27 of 51 subjects (52.9%) died because of a localized or systemic single bacterial infection. Interestingly, *E. coli* was involved in 15 cases and *haemolytic E. coli* in 4 newborn puppies, *β-haemolytic streptococci* were isolated in 3 subjects, *E. faecalis* was found in 2 cases, whereas in 3 newborn puppies were detected *P. mirabilis*, *K. pneumoniae* and *S. aureus*, respectively.

In 6 of 51 subjects (11.8%) the death was probably due to a bacterial co-infection. In the first newborn puppy the association between *E. coli* and *K. pneumoniae* was isolated in the kidney, liver, and small bowel, in addition to *Aerococcus viridans* in the lung and thoracic effusion, whereas in the second subject *E. coli* and *K. pneumoniae* were identified in the kidney, liver, and abdominal

effusion. In the third neonate, the lethal association was represented by *K. pneumoniae* in the kidney and *E. coli* in the lung, whereas in the fourth subject death might be attributed to *P. mirabilis* in the kidney, liver, and small bowel, *P. aeruginosa* in the small bowel, and *S. aureus* in the lung. In the fifth case the newborn puppy probably died because of *E. coli* in all organic samples and abdominal effusion, in addition to *E. faecalis* in the liver, whereas in the sixth newborn the mortality could be due to *haemolytic E. coli* in the kidney, lung, and small bowel, and *E. faecalis* in the lung.

Of the 51 newborn puppies, 6 (11.8%) revealed only physiologic bacteria in the small bowel, and 12 (23.5%) were characterized by having all the organic samples negative at bacteriological examination.

The AST was performed in 33 cases: the most effective drugs were third generation cephalosporins (25 cases, 75.7%) and fluoroquinolones (20 cases, 60.6%). In 29 cases (87.9%), a multidrug resistance (resistance to at least 4 antibiotics) of the bacterial strains was noted, whereas in 2 cases (6.1%) cases the isolated bacteria were resistant to all the tested antibiotics.

15.4.4 Histology

Histological examination evidenced a multi-organ morphology characterized by typical findings of an on-going maturation process. Despite gross lesions were detected during necropsy in only 8 of 51 puppies, noteworthy histological findings were found in 16 cases (31.4%). The most affected organ was the lung (12 cases, 75%) and the lesions included fibrino-purulent, purulent and necrotizing bronchopneumonia or pneumonia (5), necrosis (3), and oedema (6). In particular, 3 newborn puppies showed more than one of these pulmonary alterations simultaneously. In 4 subjects (25%) only, or also, the kidney was affected by some lesions, such as cortical or tubular necrosis (3) and acute infarctions (1). Furthermore, in 2 newborn puppies (12.5%) the liver was characterized by multifocal necrosis. However, bacterial aggregates were never detected. Hyperemia was found in all the samples belonging to 9 of the 51 puppies (17.6%).

15.5 Discussion

Despite the high percentage of neonatal death rate in dog, in the last decades few studies investigated the role of bacterial infections in newborn puppies mortality (7, 27, 31, 32, 37).

Neonatal mortality rate is underestimated by both owners and breeders, as proved by the about 20% of bitches which have experienced neonatal loss, whose causes have not been investigated. In over 70% of cases, multiple puppies in the same litter were affected by neonatal diseases, showing that the breeders should consider with great attention also the first sign of sickness even in a single newborn. In agreement with extant literature, also in the present study, clinical signs, when present, were not specific, abrupt, and were followed by fast and fatal clinical course, not allowing a possible patient treatment (8, 16, 37).

Post-mortem examination confirmed the limited value of necropsy alone; indeed, in most cases gross organic lesions were not or minimally detectable, as previously reported (7, 19, 33, 37). The usefulness of necropsy is to allow the collection of samples for bacteriology, AST, and, at a less extent, histology.

On a total of 214 collected swabs, more than 47% was bacteriological positive. It is reasonable to believe that in 65% of puppies bacterial infection might have been involved in neonatal death, confirming that bacteria could play an important role in canine neonatal mortality (19, 33). In about 23% of newborns all the swabs were bacteriological negative, whereas in about 12% of cases only physiological bacteria were isolated in the small bowel.

Literature reports that the bacterial organisms most frequently responsible for neonatal mortality in dogs are *E. coli*, staphylococci, streptococci, and *Klebsiella* spp.; however, *P. mirabilis* and *P. aeruginosa* can be also isolated (2, 7, 8, 11, 16, 25, 26, 32, 33, 37). The results of this study confirmed that the just mentioned bacteria, alone or in association, are often involved in neonatal death in dogs, above all *E. coli* (19 of 51 cases). The newborn puppy enteric epithelium is more permeable to *E.coli* than the adult one (38) and the most important infection sources are represented by mother (2, 3, 29), or other subjects living in the kennel, as well as the environment (26). Among staphylococci, the principal pathogenic species are thought to be *S. aureus* and *S. pseudintermedius*. Despite the former usually being described as frequent cause of neonatal septicaemia (27, 32), in the present study *S. aureus* was probably responsible for neonatal death in only 1 case. Streptococcal septicaemia is another common cause of miscarriage

or neonatal loss in the dog (11, 17, 18, 26), nevertheless in this research only 3 newborn puppies died because of *β-haemolytic streptococci* infection. Also streptococci often have a maternal origin (2, 3, 36); the consumption of contamined milk represents another possible, but unusual, source of infection (33). Regarding *K. pneumoniae*, it was likely responsible for neonatal loss in only 1 case, as well as *P. mirabilis*. The use of antibiotics that reduce resistance to colonization, nutrition with commercial formulas, and iatrogenic transmission seem to predispose the newborn puppy to *K. pneumoniae* infection.

In 4 puppies it was supposed that less frequent, but potentially pathogenic bacteria, such as *E. faecalis* (2), *A. viridans* (1), and *P. aeruginosa* (1), contributed to the neonatal death. *Enterococcus faecalis* is a bacterium belonging to the normal endogenous flora of humans and animals but its intestinal excess or systemic diffusion represent a real problem, above all in the newborn. *Aerococcus viridans* is a Gram-positive coccus rarely found as human pathogen (20), but in literature it has been reported that, in vulnerable patients, this organism could have a clinically significant role in systemic infections (34); this fact might happen also in canine species. *Pseudomonas aeruginosa* is frequently involved in diseases caused primarily by other bacteria; immunosuppressive or too long antibiotics therapies are predisposing factors for the infection.

In 14 of 101 positive samples, bacterial associations were isolated, as already reported by other authors (33), and in 6 out of 51 newborn puppies death was probably due to the bacterial co-infection.

Obviously, other possible causes of mortality should be considered in those cases in which all the swabs were bacteriological negative (12) or only physiological bacteria were isolated in the small bowel (6).

Beyond bacterial detection, the present study was also aimed to assess antibiotic sensitivity of isolated bacteria. In 33 cases the antimicrobial susceptibility was tested: the most effective drugs were third generation cephalosporins, in agreement with data reported by other authors (7, 8, 16, 26, 37), and fluoroquinolones. In 88% of cases the bacterial strains showed a multidrug resistance, and in 6% of cases the bacteria were resistant to all the tested antibiotics. It has been already demonstrated that bacterial antimicrobial resistance and multiresistance represent an emerging problem (12). The spread use of antibiotics by many breeders to reduce the neonatal mortality might be responsible for the dam vagina colonization by opportunistic pathogens and the selection of resistant bacteria, which may cause septicaemia in newborn puppies

(21). Therefore, the AST should be strongly recommended after bacterial detection, to optimize the efficacy of therapy and to avoid dangerous bacterial resistance (7, 8, 37).

Histology, reported as a useful tool for septicaemia diagnosis, was of limited value in the present study. Indeed, bacterial emboli within the blood vessels or internal organs, or bacterial growth within the deep tissues (7, 9) were never seen, in contrast to what reported by others authors (19, 36). A possible explanation for this finding could be the fast course of bacterial infection in newborn puppies, so that the neonates died before the establishment of the typical histological changes (24).

The present study demonstrates the involvement of bacterial infections in neonatal mortality in canine species and the alarming antibiotic resistance of the isolated bacterial strains.

Neonatal loss should not be underestimated by owners and breeders, and necropsy, coupled to bacteriological examination and AST, should be always suggested in even isolated neonatal mortality occurrence.

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CHAPTER 16

Age estimation

in large and giant newborn puppies

through

the hindlimb ossification centers evaluation

and

morphometry

of the hindlimb long bones, skull, and body

16.1 Introduction

In humans, several skeletal segments were studied as markers for age estimation. In particular, in dead human beings, the cranial suture closure, dentition, and epiphyseal closure represent the more commonly examined areas, whereas in children and adolescents, the teeth mineralization status, long bones diaphyseal lenght, and developmental status of the epiphysis are usually studied (Schmeling *et al.*, 2007; Cunha *et al.*, 2009; Boyne *et al.*, 2010; Franklin, 2010). However, to date the radiographic exam of the left hand holds a primary role in the estimation of skeletal age in living people, until the end of skeletal maturation (Cunha *et al.*, 2009; Schmidt *et al.*, 2013b).

Generally, also both the appearance and fusion of the ossification centers (OCs) can provide some important information to estimate the age in dead as well as in living people (Cunha *et al.*, 2009; Schmidt *et al.*, 2013a). At this regard, some researches reported the Dual-energy X-ray Absorptiometry (DEXA) as a useful tool to investigate the OCs appearance (Panattoni *et al.*, 1999; Panattoni *et al.*, 2000). Unfortunately, it was demonstrated that the growth pattern depends both on the ethnicity and age, and it could be affected by nutritional and individual factors; thus, often there is not a perfect correspondence between the biological and the chronological age (Cunha *et al.*, 2009).

As in humans, generalization about normal growth are difficult to make in canine species, mainly due to the wide variety of breeds encompassing many body shapes and sizes. The dog is one of the few species characterized by a large variety of breed sizes, which makes metabolic and growth rates significantly different. Breed-specific differences in growth patterns might be expected because of the huge variations in size, temperament, and coat type, which are able to affect the energy requirements (Hawthorne *et al.*, 2004), as reported by Rainbird and Kienzle (1990). Little information were published on breed-specific changes in puppies growth patterns; furthermore, most studies documented only limited data on single breeds, such as 8-34 month old and 6-20 weeks old Labrador Retrievers (Alexander and Wood, 1987; Booles *et al.*, 1994), as well as 0-12 weeks old and 0-7 weeks old German Shepherds (Schroeder and Smith, 1994; Elmaz *et al.*, 2008).

The growth and weight gain represent an important indicator of health in puppies, above all within the neonatal period, but they imply a gradual development of the whole skeletal system.

In mammals, two distinct ossification processes occur simultaneously: the intramembranous ossification, which produces many craniofacial bones directly from mesenchymal condensations (Percival and Richtsmeier, 2013), and the endochondral ossification, that gives rise to the most skeletal bones (Mackie *et al.*, 2011). Particularly, endochondral bone growth starts with the proliferation, maturation, and hypertrophy of chondrocytes, organized in OCs, to allow the mineralization of cartilaginous matrix to form an osseous tissue (Panattoni *et al.*, 1999; Wongdee *et al.*, 2012). Endochondral ossification begins during the intrauterine life and continues until the early adulthood, whereas the development of the secondary OCs generally occurs after birth (Evans and de Lahunta, 2013).

In dogs, it is well-known that limbs bones develop from one or more OCs, and both their appearance and fusion occur within different timing. Skeleton of growing dogs was investigated by morphometric, radiographic, photodensitometric, and bone mineral density (BMD) studies aimed to establish breed standard (Onar, 1999; Onar and Günes, 2003; Driver *et al.*, 2010; Schmidt *et al.*, 2011), to investigate abnormal skeletal development (Breit *et al.*, 2004; Vanden Berg-Foels *et al.*, 2006) and to exclude some pathologies (Todhunter *et al.*, 1997; Emmerson *et al.*, 2000; Meomartino *et al.*, 2002; Mostafa *et al.*, 2009; Doskarova *et al.*, 2010; Vanden Berg-Foels *et al.,* 2011), to quantify the long bone healing (Zotti *et al.*, 2004), to assess BMD variations in different breeds (Markel *et al.*, 1994), and to evaluate the resistance of canine spine to traumatic lesions (Zotti *et al.*, 2011). Many papers were aimed to estimate the age in growing dogs by evaluating the OCs. Most of them enrolled medium and large breed dogs, as Beagles (Hare, 1961; Chapman, 1965; Yonamine *et al.*, 1980; Mahler and Havet, 1999), German Shepherds (Hare, 1961; Gustaffson *et al.*, 1975; Charjan *et al.*, 2002; Elmaz *et al.*, 2008), and Greyhounds (Smith, 1960a-b, 1964; Gustaffson *et al.*, 1975; Riser, 1975). Nevertheless, it is really difficult to compare the respective findings because in most cases the methods, breeds, age of examination, anatomical compartments investigated, as well as the research purposes, appeared not homogeneous.

Regarding the morphometry, only few indications were documented about the morphometric changes of skeleton and BMD in dogs (Delaquerriere-Richardson *et al.*, 1982; Helmsmuller *et al.*, 2013). Furthermore, studies about the employment of DEXA to investigate the OCs appearance are really scarce in veterinary medicine.

Aim of the study

At present, at least in Italy, the illegal import of puppies represent an emerging problem. Based on D. Lgs. 576/2013, in Italy the import of puppies younger than 3 months and 21 days is allowed without the dam and without a valid vaccination for rabies only with a certificate demonstrating that, from the birth to the movement, there was no contact between the subject and other animals affected by rabies. In our country, the obligation to move puppies at least 3 months and 21 days old is mainly due to the fact that the vaccine against rabies can be performed from 3 months of age on forward and it becomes protective 3 weeks after the administration. However, some countries allow to perform this vaccination early, by using expressely registered vaccins, since the European Community did not impose any specific age to start the vaccination against rabies. Indeed, in these cases, the competent authorities of the puppies home town should declare and certify on the animal passport the possible employment of a specific vaccine which can be used in subjects younger than 3 months of age.

Although the strict supervision, the illegal import of puppies often too young continues, especially from the East Europe countries. Thus, new, easily performable, non invasive methods would be necessary to estimate the biological age of illegal imported puppies. In canine species, to date the dentition examination remains the most widespread tool for age estimation in growing subjects. Nevertheless, especially in case of illegally imported puppies younger than 1 month of age, this method is not so useful since the teeth growth in dogs usually starts around 3-4 weeks of age (Veronesi, 2013). At this regard, the evaluation of limbs OCs appearance and the morphometric measurements of the limbs long bones, skull, and body length could provide better guarantees. Additionally, to the author knowledge, the researches about the age estimation by evaluating the OCs and morphometry are scarce in dog, above all within the first month of age.

Thus, the present study was aimed to: 1) evaluate the chronological appearance of the OCs in hindlimb of large-giant sized purebred puppies, dead spontaneously within the first month of age and with a normal weight for the respective breed and age at the time of death; 2) study the morphometry of hindlimb long bones, skull, and whole body of normal newborn puppies, by both radiographic and anatomical approaches; 3) detect possible correlations among age, body weight, and radiographic measurements, as well as among age, body weight, and anatomical measures; 4) assess possible correlations among radiographic measurements themselves, as well as among anatomical ones; 5) found possible correlations between radiographic and anatomical measurements; 6) evidence possible correlations among femoral BMD, age, and body weight, as well as between femoral BMD and radiographic measurements, and between femoral BMD and anatomical ones; 7) evaluate and quantify the trends of the ossification process and the architectural changes of OCs, through histological sections of hindlimb long bones.

16.2 Materials and methods

The present study enrolled 79 newborn puppies, 43 males and 36 females, belonging to several large and giant size breeds, dead spontaneously within 30 days of age. The respective owners signed an informed consent for the use of each cadaver for research purposes. After death, the subjects were weighted, immediately chilled at 4°C, and stored for at maximum 12 hours. Radiographic projections, densitometric analysis, radiographic and anatomical measurements, as well as long bones histological sampling were performed as soon as possible, always within 24 hours after death for each puppy.

Based on the age-at-death, all the newborns were grouped as follows: Group 1 (premature), Group 2 (born dead to 6 days of age), Group 3 (7 to 14 days of age), Group 3 (15 to 25 days of age), and Group 4 (26 to 30 days of age). The subjects were classified as premature when they were delivered between 2 and 1 weeks before the expected date of whelping, estimated by ultrasonographic parturition date prediction (Veronesi, 2013).

16.2.1 Radiographic examination

Radiographic studies were performed by using two CR systems (Agfa ADC COMPACT® e FCR Fuji Capsula X) assembled with a radiological unit

(ARCOM-Simply) with double focal spot (0.6 and 1.3 mm), 32 Kw of nominal anode input power and inherent filtration of 0.7 mm Al eq. The focal spot-film distance was 100 cm and the central ray was perpendicular to the film in all the radiographs. For every newborn puppy, several radiographic projections were performed: latero-lateral (LL) and dorso-ventral (DV) of the head, medio-lateral (ML) and caudo-cranial (Cd-Cr) of the hindlimb, as well as latero-lateral (LL) and dorso-ventral (DV) of the whole body.

Latero-lateral views of both the head and whole body were obtained placing the dogs on left lateral recumbency with the forelimbs and the hindlimbs superimposed, by fixing them at the radiographic cassettes with radiolucent adhesive tape. The head was placed in lateral position. Dorso-ventral views were performed placing the subjects on ventral recumbency, with the flexed arms externally in contact with the radiographic cassettes. Medio-lateral views of the hindlimb were obtained by placing the dogs on the side of the radiographed limb; the controlateral one was carefully moved cranially, superimposed with the body, and fixed with radiolucent adhesive tape. Caudo-cranial views were performed placing the subjects on ventral recumbency, extending the radiographed hindlimb caudally and fixing it at the radiographic cassettes with radiolucent adhesive tape.

All images were stored in an Apple data base and both post-processing evaluation and radiographic measurements were performed by OsiriXPRO software (Apple®).

16.2.2 Ossification centers evaluation

The appearance of a radiopaque area on radiographs at the level of the corresponding bone was considered as the standard for OC evaluation (Hare, 1959b). The following hindlimb OCs were considered in the 79 subjects examined: ilium (*os ilium*), ischium (*os ischii*), pubis (*os pubis*), proximal and distal epiphyses of femur (*os femoris*) and tibia (*tibia*), fibula, patella, and tarsal bones (*ossa tarsi*).

16.2.3 Radiographic measurements of the hindlimb long bones, skull, and body length

The length of the hindlimb long bones, skull, and body was measured on radiographs in all the 79 newborns enrolled. Measurements of femoral and tibial lengths were taken on the ML projections, between the most proximal and distal

points of the long bones (Alpak *et al.*, 2004). At birth, only the long bones diaphysis appear radiopaque, thus in newborn puppies the long bones length corresponds to the length of diaphysis (Riser, 1973).

On the LL projection of the head, the skull length (SL) was measured, whereas the measurements of neurocranium width (NW) and zygomatic width (ZW) were taken on the DV projection of the head. Particularly, the SL was measured both from the anterior end of the interincisive suture to the external occipital protuberance (SL1), and from the anterior end of the interincisive suture to the aboral border of the occipital condyles (SL2). The NW was measured from the most lateral point of the brain case to the one of the other side, whereas the ZW from the most lateral point of the zygomatic arch to the most lateral point of the other (Onar, 1999; Onar and Günes, 2003; Alpak *et al.*, 2004).

The vertebral column length (VCL) was measured on the LL projection of the whole body, from the first cervical vertebra to the ischiatic tuberosity (Evans and de Lahunta, 2013).

In order to reduce the inaccuracies, each measurement was performed three times by the same single operator.

16.2.4 Densitometric analysis

Densitometric analysis was performed only on 10 subjects out of 79; among these, 2 newborn puppies belonged to Group 1, 3 subjects to Group 2, 2 puppies to Group 3, 2 to Group 4, and only 1 to Group 5. All the subjects were scanned by mean of a DEXA device (Hologic QDR-1000, Hologic, Waltham, MA, USA). The cadaver was scanned standing horizontally, with a disto-proximal direction, and in a medio-lateral projection. Before scanning, the unit was always calibrated by mean of own calibration phantom (Hologic Calibration Phantom, Hologic). The general BMD of the femur was calculated and it was expressed as grams of bone mineral on the scanned site area (g/cm^2) (Panattoni *et al.*, 1999; Zotti *et al.*, 2011). Each scan was performed by the same single operator.

16.2.5 Anatomical measurements of hindlimb long bones, skull, and body length

Anatomical measures of the hindlimb long bones, skull, and body length were taken only on the 10 puppies selected for the densitometric examination. Before

the skeletonization of the hindlimb, femoral and tibial lengths were measured using a caliber, with and without the skin, and finally after the skeletonization. Measures were carried out on the lateral sides of each limb examined. The femoral length was measured between the most proximal and distal points of the bone, as well as the tibial length (Alpak *et al.*, 2004).

The skull (Alpak *et al.*, 2004) and VCL (Evans and de Lahunta, 2013) measurements were performed using a caliber, without skeletonization of the cadavers.

In order to reduce the inaccuracies, each measurement was taken three times by the same single operator.

16.2.6 Histological analysis

Histological analysis was performed only on 9 out of those 10 subjects selected for anatomical measurements. After the hindlimb skeletonization, proximal and distal epiphysis of femur, calcaneus, talus, and IV tarsal bone were sampled for the histological examination. All the samples were immediately fixed in buffered 10% formalin (Bio-Optica, Milan, Italy) for 2-3 days; then, they were decalcified firstly with 45% formic acid (Sigma Chemical Company, St. Louis, USA) for 2-3 days at 4°C, and later with 15% 0.5M EDTA solution (Sigma Chemical Company, St. Louis, USA) (pH 8.0) for other 7 days at 4°C, according to Ozaki *et al.* (2010) with a slight modification. Finally, the samples were dehydrated in graded alcohol and xylene series, and embedded in paraffin. Serial sections (4 μm) were mounted on the glass slides, previously treated with Vectabond (Vector Laboratories, Burlingame, CA, USA) to enhance the tissue adherence, and they were stained with hematoxilin-eosin and Thichrome Staining (Masson-Bio-Optica).

The OCs detectable by histological examination were classified in type 0 (OCT0), 1 (OCT1), 2 (OCT2), and 3 (OCT3).

16.2.7 Statistical analysis

Statistical analysis was performed with the SPSS 20.0 (SPSS Inc., Chicago, IL, USA). In the present study, the data were presented as mean±standard deviation (SD). Firstly, the Pearson correlation coefficient was employed to assess the following possible correlations: 1) among age, body weight, and radiographic

measurements of hindlimb long bones, skull, and body length, of the 79 subjects, as well as 2) among radiographic measurements themselves belonging to the 79 newborn puppies. The significance level was set at $P<0.01$. Secondly, the Covariance analysis (ANCOVA) was performed to show any possible influence of gender, age, and body weight, on each radiographic measurement, considering both the gender and age as categoric variables, whereas the body weight as covariate. Furthermore, the Tukey test was used to investigate the possible differences among the mean values of the different classes of age. The significance level was set at $P<0.05$. Then, a multiple regression of all the variables considered towards the age was performed by using Foreward Stepwise program in order to find an equation, based on those measurements most significantly correlated with age, to estimate the real puppies age.

Since the densitometric analysis, as well as the anatomical measurements, were performed only on 10 and 9 subjects out of the 79, respectively, the data distribution resulted not normal; thus, a non parametric Rho Spearman test was carried out to assess the following possible correlations: 1) between femoral BMD and both age and body weigth, 2) between femoral BMD and radiographic measurements, and 3) between femoral BMD and anatomical measurements. The significance level was set at $P<0.05$.

Furthermore, the non parametric Rho Spearman test was also employed to assess the following possible correlations: 1) among anatomical measurements themselves, 2) between anatomical measurements and both age and body weight, and 3) between anatomical and radiographic measurements. The significance level was set at $P<0.05$.

16.3 Results

In the present study 79 purebred puppies, belonging to large and giant size breeds (25kg<large<40kg; giant>40kg), 36 females and 43 males, dead spontaneously within 30 days of age, were enrolled. Particularly, 14 different breeds were involved: Bull Mastiff (n=28), Labrador Retrievers (n=10), Golden Retrievers (n=1), Boxer (n=4), Afghan Hound (n=5), German Shepherd (n=3), Belgian Shepherd (n=1), Giant Schnauzer (n=5), Hovawart (n=1), Maremma (n=6), Rottweiler (n=6), Saint Bernard (n=1), Leonberger (n=7), and Alaskan Malamute (n=1).

The Group 1 (premature) included 18 puppies, the Group 2 (born dead to 6 days of age) 51 subjects, the Group 3 (7 to 14 days of age) was composed by 6 puppies, whereas both the Group 4 (from 15 to 25 days of age) and Group 5 (from 26 to 30 days of age) included only 2 subjects. The newborns were not grouped based on the week of age in the attempt to increase the extent of some classes of age and, however, on the basis of some skeletal resemblances.

16.3.1 Radiographic evaluation of the ossification centers

The diaphysis of all the hindlimb long bones were identified by radiographic examination already after birth, also in premature puppies (Fig.1), and no differences were macroscopically detected between right and left hindlimbs. The distribution of the hindlimb OCs percentage of radiographic detection, according to the group of age, in the 79 examined puppies is shown in Table 1 (Fig.2-7).

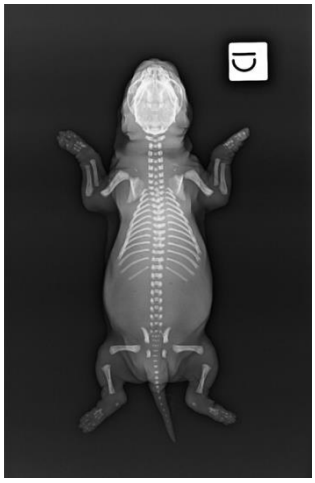

Figure 1- Dorso-ventral projection of the whole body in a premature newborn puppy.

Long bones diaphysis are already present.

OCs/Age	Group 1 (n=18)	Group 2 (n=51)	Group 3 (n=6)	Group 4 (n=2)	Group 5 (n=2)
Ischium	100%	100%	100%	100%	100%
Ilium	100%	100%	100%	100%	100%
Pubis	44.44%	96.08%	100%	100%	100%
Femoral diaphysis	100%	100%	100%	100%	100%
Femoral head	0%	1.96%	16.67%	100%	100%
Greater trochanter	0%	0%	0%	0%	0%
Lesser trochanter	0%	0%	0%	0%	0%
Femoral trochlea	0%	1.96%	0%	100%	100%
Medial condyle	0%	0%	0%	100%	100%
Lateral condyle	0%	0%	0%	100%	100%
Patella	0%	0%	0%	0%	0%
Tibial diaphysis	100%	100%	100%	100%	100%
Proximal epiphysis	0%	0%	0%	50%	100%
Tibial tuberosity	0%	0%	0%	0%	0%
Cochlea	0%	0%	0%	50%	100%
Medial malleolus	0%	0%	0%	0%	0%
Calcaneus	100%	100%	100%	100%	100%
Talus	0%	90.20%	100%	100%	100%
Central tarsal bone	0%	0%	0%	50%	100%
I tarsal bone	0%	0%	0%	0%	100%
II tarsal bone	0%	0%	0%	0%	100%
III tarsal bone	0%	0%	0%	50%	100%
IV tarsal bone	0%	3.92%	16.67%	100%	100%

Table 1-Distribution of the radiographic detection percentage of the hindlimb OCs in the 79 examined large and giant sized puppies, according to the group of age

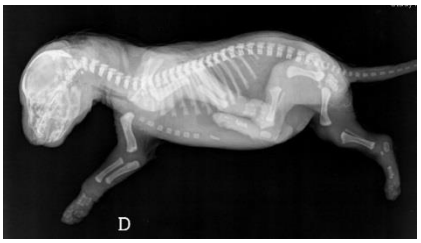

Figure 2- Latero-lateral projection of the whole body in a born dead newborn puppy.

The calcaneus and talus are already detectable.

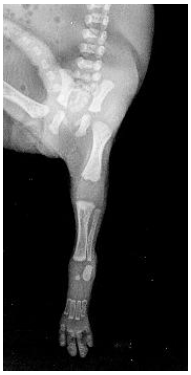

Figure 3- Caudo-cranial projection of the hindlimb in a 7-days old puppy.

Calcaneus and talus are visible, beyond the pubis in the pelvis.

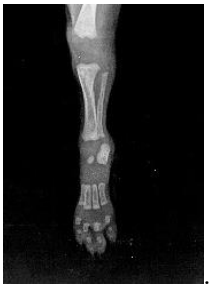

Figure 4- Caudo-cranial projection of the hindlimb in a 14-days old puppy.

Calcaneus and talus appear more developed.

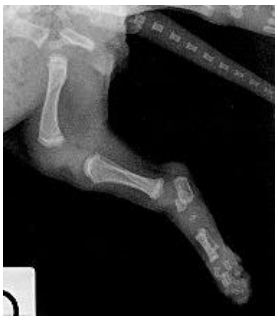

Figure 5- Latero-lateral projection of the hindlimb in a 21-days old puppy.

The femoral head and IV tarsal bone are detectable.

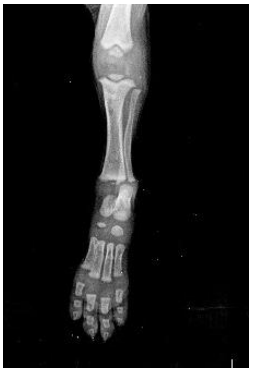

Figure 6- Caudo-cranial projection of the hindlimb in a 25-days old puppy.

Femoral distal epiphysis, tibial proximal epiphysis, central and IV tarsal bones become visible.

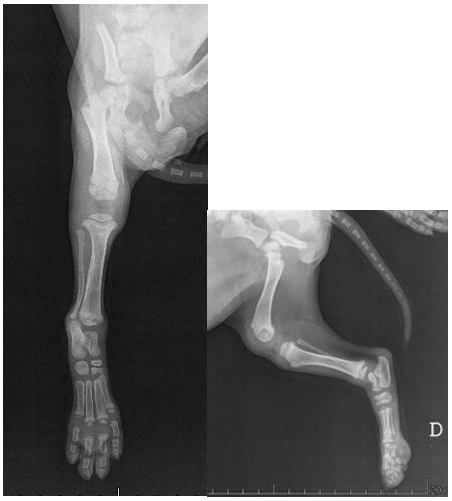

Figure 7- Hindlimb in a 30-days old puppy. All the major hindlimb OCs are detectable.

16.3.2 Radiographic measurements

All the mean values (±SD) of the skull, body, and hindlimb long bones lengths (expressed in cm) in the 79 subjects are summarized in Table 2.

Measurement (cm)/Age	**Group 1 (n=18)**	**Group 2 (n=51)**	**Group 3 (n=6)**	**Group 4 (n=2)**	**Group 5 (n=2)**
Skull length 1	4.29±0.26	5.33±0.55	5.48±0.51	7.13±0.92	9.65±0.52
Skull length 2	4±0.28	5.09±0.56	5.21±0.38	6.61±0.70	9.12±0.46
Neurocranium width	2.55±0.23	2.95±0.27	3.14±0.23	4.28±0.19	5.27±0.18
Zygomatic width	3.41±0.17	3.76±0.37	4.11±0.48	4.97±0.52	6.39±0.19
Vertebral column length	13.74±1.10	16.73±2.29	17.23±1.38	21.92±1.84	30.60±1.17
Femoral length	1.90±0.15	2.49±0.32	2.70±0.36	3.35±0.42	5.67±0.19
Tibial length	1.66±0.20	2.29±0.31	2.54±0.32	3.23±0.62	5.25±0.06

Table 2 - Mean values (±SD) of the skull, body, and hindlimb long bones lengths (expressed in cm) in the 79 large and giant size puppies, according to the group of age

All the radiographic measurements of the hindlimb long bones, skull, and body lengths resulted highly correlated ($P<0.01$) among themselves. Furthermore, it was found a significant positive correlation ($P<0.01$) among these radiographic measures, age, and bodyweight. Interestingly, the neurocranium width and tibial length were detected as the most signicantly correlated parameters with age.

The ANCOVA, followed by Tukey test, allowed to evidence a significant positive correlation ($P<0.05$) between all the radiographic measurements and both age and bodyweight. In particular, all the mean values of the radiographic measures of hindlimb long bones, skull, and body lengths appeared statistically different ($P<0.05$) in the 5 groups of age, except the mean values of the neurocranium width, skull length, vertebral column length, as well as both femoral and tibial lengths, which did not seem statistically different between the Groups 2 (born dead-6 days) and 3 (7-14 days). Regarding the correlation between the bodyweight and every radiographic measurement, it was possible to

calculate a specific equation to express this correlation (Table 3). Statistic analysis failed to show any correlation between the gender and radiographic measures, except for the zygomatic width (3.87±0.74 in females vs. 3.77±0.46 in males, $P<0.05$).

Radiographic measurement	Correlation with bodyweight (BW)
Skull length 1	4.01+(0.0028*BW)
Skull length 2	3.81+(0.0026*BW)
Neurocranium width	2.27+(0.0015*BW)
Zygomatic width	3.01+(0.0018*BW)
Vertebral column length	12.28+(0.0096*BW)
Femoral length	1.62+(0.0019*BW)
Tibial length	1.46+(0.0018*BW)

Table 3 - Correlation between radiographic measures and bodyweight

The multiple regression of all the variables considered towards the age, performed by using Foreward Stepwise program, selected the neurocranium width (NW) as well as femoral (FL) and tibial lengths (TL) as the most predictive variables to estimate the newborn age. Thus, it was possible to create an equation, based on these variables, aimed to the estimation of age in large and giant sized puppies within the first month of post-natal life: age=-33,773+(NW*9,677)+(TL*21,462)-(FL*17,563) ($r=0.895$; $r^2=0.802$). In the attempt to avoid the possible influence of the bodyweight, also the latter was added in the multiple regression, changing the equation in the following way: age= -24,1717+(NW*11,479)+(TL*24,447)-(FL*17,680) ($r=0.903$; $r^2=0.816$).

16.3.3 Densitometric findings

The densitometric study was performed only on 10 subjects; among these, 2 newborn puppies belonged to Group 1, 3 subjects to Group 2, 2 puppies to Group 3, 2 to Group 4, and only 1 to Group 5. The mean values of femoral BMD were reported in Table 4. The minimum value was 0.1516 g/cm^2, whereas

the maximum was 0.3107 g/cm^2; the first value belonged to a premature newborn puppy, whereas the second one to a 30-days old puppy.

	Group 1 (n=2)	**Group 2 (n=3)**	**Group 3 (n=2)**	**Group 4 (n=2)**	**Group 5 (n=1)**
Femoral BMD (g/cm^2)	0.15	0.20±0.02	0.24±0.02	0.23±0.01	0.31

Table 4 - Mean values±SD of femoral BMD in the 10 puppies evaluated, according to the group of age

The Rho Spearman test revealed a significant positive correlation between the femoral BMD and both age and bodyweight ($P<0.01$). Additionally, it was evidenced also a significant correlation between femoral BMD and both radiographic and anatomical measurements of hindlimb long bones, skull, and body lengths ($P<0.05$).

16.3.4 Anatomical measurements

Anatomical analysis was performed on 9 out of the 10 puppies selected for densitometric examination, except the oldest one. The mean (±SD) values (cm) of the anatomical measurements of hindlimb long bones, skull, and body lengths, performed in 9 puppies are summarized in Table 5.

Measurement (cm)/Age	Group 1 (n=2)	Group 2 (n=3)	Group 3 (n=2)	Group 4 (n=2)
Skull length 1	4.47±0.75	5.45±0.48	6±0.09	7.58±0.68
Skull length 2	4.55±0.87	5.38±0.50	5.45±0.45	7.35±0.68
Neurocranium width	2.52±0.54	3.11±0.42	2.98±0.02	4.48±0.16
Zygomatic width	2.82±0.26	3.53±0.46	3.98±0.12	4.5±0.52
Vertebral column length	13.86±2.21	16.98±1.24	18.25±0.40	21.32±0.87
Femur with skin	2.73±0.09	3.61±0.60	3.73±0.19	4.22±0.07
Tibia with skin	2.4±0.38	3.02±0.30	3.53±0.05	4.32±0.49
Femur without skin	2.47±0.19	3.19±0.37	3.62±0.02	4.43±0.19
Tibia without skin	2.38±0.35	2.88±0.50	3.32±0.31	4.08±0.68
Scheletonized femur	2.63±0.42	3.28±0.35	3.4±0.09	4.35±0.40
Scheletonized tibia	2.30±0.23	3.08±0.26	3.13±0.14	4±0.56
Tarsus thickness	0.35±0.07	0.5±0.1	0.55±0.07	0.75±0.07

Table 5 - Mean (±SD) values (cm) of the anatomical measurements in the 9 puppies, according to the first 4 groups of age

Firstly, the Rho Spearman test evidenced a significant correlation ($P<0.01$) among all the anatomical measurements themselves. Secondly, it was demonstrated a significant positive correlation ($P<0.01$) between these anatomical measures and both age and bodyweight. In particular, the femoral and tibial lengths seemed to be the most correlated anatomical measures with age. Finally, the statistical analysis showed also a significant correlation between anatomical and radiographic measurements ($P<0.01$).

16.3.5 Histological findings

The histological and histochemical analysis confirmed the OCs presence of the proximal and distal epiphysis of femur, calcaneus, talus, and IV tarsal bone, previously identified by radiographs. Based on the present findings, the OCs were classified in type 1 (OCT1), 2 (OCT2), and 3 (OCT3). The histological section was able to detect also the OC of type 0 (OCT0), not identified by X-ray images. Furthermore, it was possible to describe even an earlier stage of development, defined "resting" condition. The term "resting" refers to the earliest phase of development, characterized by small lacunae, very few chondrocytes, and mytosis. In OCT0 hypertrophic lacunae, containing hypertrophic, flattened, or degenerated chondrocytes were evident, and the extracellular matrix around the lacunae had occasionally a granular aspect (Fig. 8). The OCT1 showed chondrocytes and lacunae as the OCT0, but some of them appeared empty and confluent, and their invasion by the vessel of the cartilage canals started. In the long bone, OCT1 had a spherical organization. In OCT2, most lacunae became fused togheter, delimited by septa of cartilage extracellular matrix and invased by vessels. Occasionally, it was possible to observe neo-formed bone tissue, closed to the septa (Fig. 9). In long bones, OCT2 showed a spherical organization. OCT3 was characterized by an interweaving of septa covered by neo-formed bone tissue and ossification cells (Fig. 10). Bone marrow cells began to be evident between the septa. In femoral head, the shape of center became hemispherical at this stage. In Table 6, the timing of histological OCs appearance observed in calcaneus, talus, and femur, is reported.

Age	Calcaneus	Talus	IV tarsal bone	Femoral head	Femoral trochlea	Femoral condyles
Premature	OCT3	Resting	-	-	-	-
Premature	OCT3	Resting	-	-	-	-
Born dead	OCT3	OCT0	-	-	-	-
Born dead	OCT3	OCT1-2	-	-	-	-
Born dead	OCT3	OCT1	-	-	-	-
7 days	OCT3	OCT2	-	-	-	-
14 days	OCT3	OCT3	OCT0	Resting	Resting	Resting
20 days	OCT3	OCT3	OCT1	OCT2-3	OCT2-3	OCT2-3
25 days	OCT3	OCT3	OCT3	OCT3	OCT3	OCT3

Table 6 - Timing of OCs histological appearance in calcaneus, talus, IV tarsal bone, and femur.

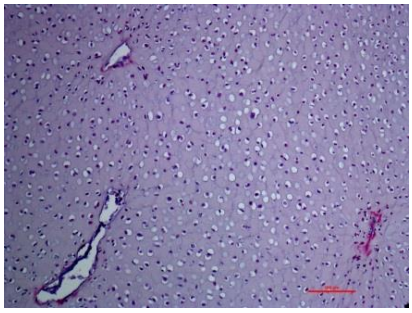

Figure 8- OCT0 in femoral head of a 7-days old puppy.

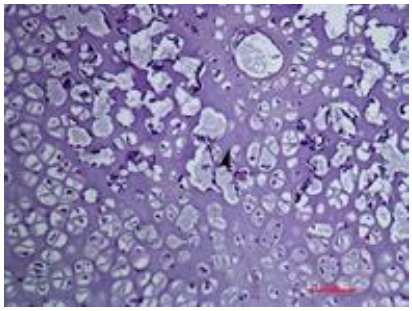

Figure 9- OCT2 in femoral head of a 20-days old puppy.

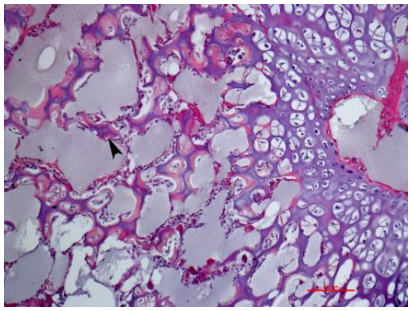

Figure 10- OCT3 in femoral head of a 25-days old puppy.

16.4 Discussion

The age estimation in human beings, as well as in dogs, represents a really common necessity. In dead people, the cranial suture closure, dentition, and epiphyseal closure are considered the best markers for this purpose, whereas in living people the radiographic examination of the left hand plays a critical role until the end of maturation process. In children, the best methods allowing the estimation of the real age seem to be the tooth mineralization, longitudinal bones length measurement, and developmental status of epiphysis (Schmeling *et al.*, 2007; Cunha *et al.*, 2009). In canine species, the dentition examination remains the most widespread tool for age estimation in growing subjects. Nevertheless, in case of puppies younger than 1 month of age, this method is not so useful since the teeth growth usually starts around 3-4 weeks of age (Veronesi, 2013). Then, the evaluation of limbs OCs appearance and the morphometric measurements of the limbs long bones, skull, and body length could provide better guarantees. Consequently, the present study was born to find objective parameters to estimate as accurately as possible the age of puppies less than 30 days old.

Firstly, all the long bones diaphysis were observed in the hindlimb since the birth (Riser, 1973), even in premature newborn puppies.

Concerning the evaluation of the OCs appearance in the pelvis, both the ischium and ilium were present at birth, as reported unanimously by the literature, whereas the pubis was not constant neither in the Group 1 (premature) nor in Group 2 (born dead-6 days) (44.44% and 96.08%, respectively), but it appeared definitely in Group 3 (7-14 days), partially in agreement with Evans and de Lahunta (2013) but in contrast to other older researches (Hare, 1960b-1961; Smith, 1964; Chapman, 1965; Ticer, 1984). The acetabular bone, iliac crest, ischiatic tuberosity, and ischial arch were not evident within the first month of age, as previously reported by Ticer (1984).

Regarding the OCs in the hindlimb, the femoral head was observed in only 1 out of 51 (1.96%) newborn puppies belonging to the Group 2 (born dead-6 days), successively in 1 out of 6 (16.67%) cases in Group 3 (7-14 days), and in 100% of the subjects in Group 4 (15-25 days), in agreement with previous studies (Hare, 1960a-1961; Chapman, 1965; Ticer, 1984; Zoetis *et al.*, 2003). Both the greater and lesser trochanters did not appear within the first month of age, as showed by

previous papers (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Fukuda and Matsuoka, 1980; Ticer, 1984; Zoetis *et al.*, 2003), whereas both the femoral trochlea and medial as well as lateral condyles became constantly evident in the Group 4 (15-25 days), in agreement with other authors (Ticer, 1984; Zoetis *et al.*, 2003). The femoral trochlea was evident in only 1 out of 51 (1.96%) cases of the Group 2 (born dead-6 days), but it was probably due to an early bone development.

The patella did not appear within the first month of age, as previously demonstrated by Hare (1960a) but contrary to Hare (1961), which reported its appearance in German Shepherds at 30 days of age.

Concerning the tibia OCs, both the proximal epiphysis and cochlea were observed in 50% (1 out of 2) of cases in the Group 4 (15-25 days) and constantly in the Group 5 (26-30 days). The appearance of the proximal epiphysis seemed to be in agreement with what reported by Fukuda and Matsuoka (1980), as well as the appearance of cochlea confirmed some previous findings (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Fukuda and Matsuoka, 1980; Zoetis *et al.*, 2003). Both the tibial tuberosity and medial malleolus did not appear within the first month of age, as demonstrated by several athors (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Fukuda and Matsuoka, 1980; Ticer, 1984; Zoetis *et al.*, 2003).

Regarding the tarsal bones, it was possible to observe the presence of calcaneus from the time of birth, even in premature subjects, confirming the data previously reported in all the breeds described (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Fukuda and Matsuoka, 1980; Ticer, 1984; Zoetis *et al.*, 2003). Talus became constant in the Group 3 (7-14 days), whereas its appearance was less homogeneous (90.20%) in the Group 2 (born dead-6 days), partially in contrast with all the previous researches, reporting that talus was always detectable at birth in medium-large sized dogs (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Fukuda and Matsuoka, 1980; Ticer, 1984; Zoetis *et al.*, 2003). Central tarsal bone appeared in 50% (1 out of 2) of the subjects in the Group 4 (15-25 days) and in 100% (2 out of 2) of cases in Group 5 (26-30 days), in agreement with Hare (1960a-1961) but in contrast to some studies documenting an earlier appearance of this bone in German Shepherd and Collie (Bressou *et al.*, 1957; Hare, 1961; Chapman, 1965). Both the I and II tarsal bones

became evident only in the Group 5 (26-30 days), confirming what suggested by Bressou *et al.* (1957). The III tarsal bone appeared in 50% (1 out of 2) of the subjects in the Group 4 (15-25 days) and constantly in the Group 5 (26-30 days), in agreement with other studies (Bressou *et al.*, 1957; Hare, 1961; Chapman, 1965; Ticer, 1984) but contrary to Hare (1960a), which reported a later appearance of it in English Bulldog. The IV tarsal bone was evident only in 3.92% (2 out of 51) of the puppies belonging to the Group 2 (born dead-6 days), in 16.67% (1 out of 6) of cases in the Group 3 (7-14 days), whereas it became constant in the Group 4 (15-25 days). All these findings seem to be in agreement with some previous studies, since different timing of appearance were reported depending on the breed examined (Bressou *et al.*, 1957; Hare, 1960a-1961; Chapman, 1965; Ticer, 1984). Thus, the present study appears in agreement with the previous literature about the OCs appearance, probably because the most previous researches enrolled medium and large breed dogs.

The real innovative contribution of the present study is represented by the morphometric measurements of hindlimb long bones, skull, and body lengths, since this topic, as well as its possible correlation with age, was scarcely investigated up to now in canine newborns. During dog gestation, some fetal morphometric measurements, performed by ultrasounds, are often employed to estimate the gestational age and the date of parturition (Beccaglia and Luvoni, 2006). However, most of the morphometric researches were performed on adult dogs, above all on canine skull, since it is the most important criterion in determining the standard breeds. In this respect, Schmidt *et al.* (2011) examined Cavalier King Charles Spaniel skull by computed tomography images and Driver *et al.* (2010) by magnetic resonance to study syringomyelia in the same breed, whereas Yildiz *et al.* (1993) and Onar *et al.* (1997) investigated German Shepherd dogs skull by anatomical approach. To the author knowledge, only few studies exist about the skull in puppies and they enrolled mainly German Shepherd dogs from 40 to 105 days of age (Onar, 1999; Onar and Günes, 2003). To date, morphometric measurements of the skull in newborn puppies are almost totally lacking, in fact only in one study (Elmaz *et al.*, 2008) German Shepherd puppies were measured at several different body sites every fourteen days from birth until weaning.

Radiographic morphometry was employed to evaluate canine hip joint through the acetabular angle of retrotorsion (Doskarova *et al.*, 2010), as well as the dorsal

acetabular rim view and the centre-edge angle (Meomartino *et al.*, 2002). In addition, Osmond *et al.* (2006) and Mostafa *et al.* (2009) examined the morphometric characteristics of the pelvic limbs in dogs with and without cranial cruciate ligament rupture. To the author knowledge, the canine skeletal development was investigated by morphometric approach only by Delaquerriere-Richardson *et al.* (1982) and Helmsmuller *et al.* (2013). Both these studies were performed on Beagle dogs but the first investigated the correlation among age, body weight, radiographic morphometrical measurements, and x-ray photodensitometry in subjects 13 and 21 months old, whereas the second monitored the ontogenetic development of dogs between 9 and 51 weeks of age.

In the present study, the radiographic measurements of hindlimb long bones, skull, and body lengths showed a highly significant positive correlation, which revealed an increase in body size parallel to the skull size, as previously demonstrated by Alpak *et al.* (2004) in small, medium, and large sized dogs. Regarding the morphometric measurements of the skull, it was possible to note that the skull length seems to increase more than the width, in agreement with Onar and Günes (2003), probably due to the fact that the study involved mainly dolichocephalic breeds. Furthermore, this research evidenced a significant positive correlation between radiographic measurements of hindlimb long bones, skull, and body lengths, and both the age and body weight. Previously, Onar (1999) suggested that all the skull morphometric measures increased with age, whereas Elmaz *et al.* (2008) documented a positive correlation among some morphological characteristics, age, and body weight. Interestingly, in the present study the age showed the highest correlation with both the neurocranium width and tibial length, measured by radiographic approach. Delaquerriere-Richardson *et al.* (1982) and Connolly *et al.* (2004) found a strong positive correlation between morphometric measurements of femur and age in dogs and in pigs, respectively. The findings of the present study are in agreement with those researches, since radiographic femoral and tibial lengths were highly correlated with age. The correlation between the neurocranium width and group of age confirmed the approach used to estimate the fetal gestational age in the second half of pregnancy in the bitch, by measuring the biparietal diameter (Beccaglia and Luvoni, 2006), suggesting that also in the first post-natal month this measure can be used for the assessment of newborn age. The mean values of the morphometric measurements of hindlimb long bones, skull, and body lengths

appeared statistically different in the 5 groups of age, in agreement with the gradual growth reported by Onar (1999), except the Group 2 and 3 which differed only for zygomatic width. This could be explained by the different number of the subjects enrolled in these two groups or it might suggest a slower development during the first 14 days of age compared to the following period. Regarding the body weight, it showed the highest correlation with the zygomatic width, even if the difference of the latter with the neurocranium width was minimal. However, it was found a correlation between the body weight and every radiographic measurement, expressed by an equation. Based on these findings, different gender did not affect the radiographic measurements, except the zygomatic width, in agreement with the literature, despite previous studies suggested significant ontogenetic differences between genders in large breeds (Helmsmuller, 2013).

Beyond the just mentioned points, the most important result of the present study was the specific correlation among the radiographic measurements of the neurocranium width and both tibial and femoral lengths, obtained by multiple regression and expressed as an equation, by which it could be possible to estimate the age of large and giant sized newborn puppies. This equation could represent an easy, quick, and non invasive tool to assess the actual age of illegally imported newborn puppies. Obviously, further researches on a larger number of animals are needed to verify the effective usefulness and the real sensitivity of this method, in large as well as in medium and small breeds.

To the author knowledge, no densitometric researches were performed to evaluate the ossification in newborn puppies, as well as the possible correlation between the long bones BMD and long bones length was not yet investigated. Several papers exist about the employment of DEXA to measure the BMD in human fetuses, neonates, and infants for an evaluation of both the skeletal development and conceptual/biological age (Braillon *et al.*, 1992; Salle *et al.*, 1992; Brunton *et al.*, 1993; Tsukahara *et al.*, 1993; Panattoni *et al.*, 1999-2000; Partyka, 2013). In canine species, DEXA was used to measure the BMD of healed femura after fracture fixation (Muir *et al.*, 1995), to check the vertebral BMD in Boxer (Zotti *et al.*, 2004), to test the canine spine resistence to traumatic lesions (Zotti *et al.*, 2011), and to study the densitometric properties of limbs (Markel *et al.*, 1994; Emmerson *et al.*, 2000). In the present study, because of the small size of the examined animals, it was not possible to choice accurately a

specific area in the metaphysis, as well as in the diaphysis, to examine the progression of the ossification process along the bone and to describe possible differences in BMD, as previously made in humans (Panattoni *et al.*, 1999-2000). Indeed, the general femoral BMD was calculated, demonstrating a significant positive correlation between femoral BMD and both age and body weight. This correlation was already documented by Panattoni *et al.* (1999) and Partyka (2013) in humans, as well as by Delaquerriere-Richardson (1982) in dogs. Furhtermore, a significant correlation was detected between the femoral BMD and both all radiographic and anatomical measurements.

From the anatomical point of view, all the anatomical measurements of the hindlimb long bones, skull, and body lengths, performed only on 9 subjects, resulted significantly correlated among themselves. Additionally, they appeared significantly and positively correlated with the age and body weight; in particular, the tibial and femoral lengths showed the highest correlation with the age, as reported by Connolly *et al.* (2004) for fetal pigs femur length and by Partyka (2013) for fetal human femur diameter. Thus, it would be very interesting to increase the number of the subjects in every class of age to verify if the tibial and/or femoral lengths, measured by an anatomical approach, could represent a more practical method to estimate the age in newborn puppies within the first month of age. Finally, it was found a significant correlation between anatomical measurements and radiographic ones.

This is the first study which described the appearance and morphological changes of the OCs in femur and some tarsal bones during the first post-natal month. All the bones samples were well preserved. The histological analysis confirmed the progression of the ossification grade inside the centers with growing age. The ossification process occurs as in other species (Burkus *et al.*, 1993; Lefebvre and Bhattaram, 2010). While in the rabbit (Rivas and Shapiro, 2002) it was demonstrated that long bone and epiphyseal development progress through sixteen structural stages, starting from 12 days-old embryos up to 18 month of age, the present study examined only the first month of age; thus, it was possible to identify only four structural stages. Based on the present study, a correlation between the histological pattern of organization and the radiographic aspect of the OCs seems to exist, according with Pazzaglia *et al.* (2011). Hypertrophic chondrocytes and enlarged lacunae (OCT1) indicated a calcium salt deposition on the cartilage matrix between cells and corresponded to the

first opacity in the middle of the cartilagineous epiphysis observed in the early phase of the OCs development. When the same OC assumed a rounded contour, it corresponded to the formation of a more structured center with calcified trabeculae and neo-formed bone tissue (OCT2-OCT3). Moreover, a further indication of the developmental change of the OCs was the variation from a spherical form (OCT2) to an hemispheric one (OCT3), more similar to the final shape of the bone epiphysis. This was evident also in radiographs, above all in femur, as reported by Yonamine *et al.* (1980). The lack of the correspondence between the morphological appearance of OCT0 and radiographic aspect was probably due to the low level of mineralization, indicating a very precocious stage of ossification.

16.5 Conclusions

The present study describes homogeneously the skeletal growth and development of large and giant breed sized puppies within the first month of age. The neonatal growth was recognized to be a gradual process, that occurs simultaneously in the body and limbs as well as in the skull. This was confirmed by the OCs radiographic and histological evaluation, the morphometric measurements of the hindlimb long bones, skull, and body by both radiographic and anatomical approaches, as well as by the densitometric analysis. Furthermore, it was verified also by the significant correlations among the morphometric findings, age, and body weight. The evaluation of OCs appearance on radiographs could be a useful tool to estimate the age in newborn puppies, even if during the first 14 days of age only minimal variations in the OCs development were detected. In this respect, the radiographic measures of the hindlimb long bones, skull, and body lengths were demonstrated to provide better guarantees; specifically, the neurocranium width, tibial and femoral lengths, measured by radiographic approach, resulted the most correlated measurements with the age. Then, they were included in a special equation that, in the future, could become the best and more practical method to estimate the age in living newborn puppies, as well as it was already done in canine fetuses with biparietal diameter to estimate the gestational age. Obviously, further studies are required to verify the reliability of this equation, by increasing the number of the subjects enrolled. Furthermore, it would be interesting to investigate if a similar equation for the age estimation can be create on the basis of some anatomical measurements; so, even the radiographic examination could

be avoided, making as practical as possible the procedures performed for the age estimation in newborn puppy.

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CHAPTER 17

General discussion and conclusions

17. General discussion and conclusions

Because of the increasing interest in small animals neonatology and the scarce knowledge about the fetal fluids features of the bitch, contrary to humans and other domestic species, the first aim of the present thesis was to investigate the characteristics of the fetal fluids belonging to normal, viable, and well developed newborn puppies born by normal term gestations. This was performed in the attempt to find another non invasive method, beyond the ultrasounds examination, to monitor the fetal and neonatal well-being.

The **first study** documented that IGF-I and NEFA concentrations are detectable in both amniotic and allantoic fluids of canine species. At physiological term pregnancy, IGF-I levels were higher in amniotic than in allantoic fluid, independently by the breed size, suggesting a fetal contribution to the amniotic IGF-I concentrations. Furthermore, IGF-I levels appeared significantly higher in amniotic fluid collected from fetuses belonging to large breeds compared to small and medium ones, demonstrating that amniotic IGF-I could be used as an indicator of growth potential also in dogs. Finally, a significant effect of the bitch on IGF-I concentrations was reported in both fetal fluids; thus, the major contribution to IGF-I fetal fluids composition could derive from the maternal compartment, or from the interaction of maternal and paternal effects. Concerning on NEFA, significant differences were noted in both fluids when breed size was considered, with higher values in amniotic and allantoic fluids belonging to small size dogs than in medium and large breeds, suggesting that probably, also in dogs, amniotic NEFA could be used as a marker of fat mobilization in response to an energy request. Further researches are needed to verify the potential relationship between IGF-I and NEFA fetal fluids levels and fetal or maternal pathologic conditions.

The **second study** demonstrated the detectability of IgG and lysozyme in canine amniotic and allantoic fluids at term pregnancy, documenting also in the dog the essential role of both fluids in the fetal protection, not only from a mechanical perspective. A high inter-individual variation was detected for amniotic and allantoic fluids, likely depending on the maternal concentrations of immunoglobulins and the rate of transplacental transfer. Despite the different type of placenta between dogs and humans, the amniotic IgG mean level was similar to that documented for the women, with IgG concentration representing

about 1/100 of the serum level of adult individuals. Lower IgG concentrations were detected in the allantoic compared to the amniotic fluid, probably due to a direct fetal IgG production. A significant effect of the maternal parity, but not of the breed body size, was observed, whereas the newborn gender was not associated to different IgG or lysozyme amniotic or allantoic concentrations. Concerning the lysozyme, its levels in fetal fluids appeared similar to the lower concentrations reported in the serum of adult dogs. Although no significant differences were found in lysozyme levels between the two fluids, it is reasonable to suppose that, even in the dog, lysozyme could work in fetal fluids similarly to what suggested for humans, providing a first line of defense against infectious agents. Since the significant contributions of fetal fluids to fetal and neonatal health, additional research is needed to better elucidate both the origin of IgG and lysozyme and the factors influencing the wide inter-individual variations.

Given that the non invasive techniques are considered more advisable to study the fetal and neonatal well-being and development, beyond the fetal fluids, the hair and nails become the newest biological matrices in which hormonal concentrations measurement is possible. Hair and nails cortisol levels represent the perfect biomarker to monitor the fetal and neonatal HPA axis activity over long periods, as hair and nails accumulate gradually this hormone from blood, providing a retrospective picture of previous long-term hormonal accumulation. Since little is known about hair cortisol analysis in canine species, whereas the nails cortisol concentrations assessment was never performed, the third aim of this thesis was to verify the cortisol levels detectability in both hair and nails belonging to normal dead newborn puppies. The **third study** documented that C is quantifiable in hair and nails of newborn dogs, thus both matrices could be interesting for non invasive, long time-frame fetal and neonatal studies also in canine species. The most relevant result was the significant effect of the class of age on both hair and nails C concentrations, with higher values in premature puppies compared to term-born dead puppies or puppies dead within the first 30 days of age. Newborn gender, breed body size and, relatively to the hair, the coat colour do not affect hair or nails C accumulation during the fetal development or the first month after birth. Further researches become essential to investigate the maternal or fetal origin (or both) of this hormone, as well as the influence of gender, breed size, or coat colour in normal puppies. Additionally, also the different pattern of C accumulation between the hair and nails within different

classes of age could be an interesting topic to investigate much deeper. Finally, further study would be obviously required to better investigate the hormonal levels in case of prematurity or fetal diseases, as well as to study its variations in both healthy and sick puppies during the neonatal and pediatric periods. The employment of these new biological matrices opens new perspectives for the study of all the maternal and fetal factors that could affect fetal and/or neonatal development and well-being

After the protective intrauterine life, the newborn has to face the dangerous world outside. In dog, the neonatal period represents a phase of high susceptibility to bacterial infection, since the immune competence is not fully reached until several weeks after birth. Indeed, the septicaemia was identified as the main cause of neonatal death during the first 2-3 weeks of age and it can show a hyperacute or subacute course. In both cases, the medical management of the infected newborn puppy is really difficult because of the sudden onset of unspecific clinical signs and the fast disease course. Despite the high percentage of neonatal losses in canine species, in the last years only few studies documented the role of bacterial infections in newborn puppies mortality. For this reason, the fourth aim of the present thesis was to clarify the real involvement of bacterial infections in canine neonatal mortality, beyond to evaluate the antibiotics susceptibility of the isolated bacteria to improve the clinical management of both litter and dam in case of neonatal septicaemia. The **fourth study** confirmed that bacteria play an important role in canine neonatal mortality, since it is reasonable to believe that in 65% of puppies bacterial infection might have been involved in neonatal death. *E. coli*, staphylococci, streptococci, and *Klebsiella* spp., but also *P. mirabilis* and *P. aeruginosa* were judged the organisms most frequently involved, alone or in association, in neonatal losses, above all *E. coli*. In some cases, less common but potentially pathogenic bacteria were isolated, such as *E. faecalis* and *A. viridans*. Based on the antimicrobial susceptibility test, the most effective drugs were third generation cephalosporins and fluoroquinolones, even if the bacterial antibiotic resistance and multiresistance represent an emerging problem.

In canine species, also the data available about the skeletal development in the neonatal period are limited. Thus, the present research was aimed to find new, easily performable methods to estimate the biological age of the puppies, often

illegally imported younger than 2 months of age, and to investigate the normal growth. Particularly, the last aim of the present thesis was to verify the timing appearance of the hindlimb ossification centers, as well as the radiographic and anatomical morphometry of the hindlimb long bones, skull, and body, in large and giant sized puppies dead spontaneously within the first 30 days of age.

The **fifth study** showed that the neonatal growth occurs gradually as the age progresses and simultaneously in the body, limbs, and skull. This was confirmed by the radiographic and histological ossification centers evaluation, the morphometric measurements of the hindlimb long bones, skull, and body, as well as by densitometric analysis. Furthermore, it was testified also by the significant correlations among the morphometry, age, and body weight. The evaluation of OCs appearance on radiographs could be a useful tool to estimate the neonatal age in puppies, even if in the first 14 days of age only few changes can be observed. On the contrary, the radiographic measures of the hindlimb long bones, skull, and body lengths seem to provide better guarantees; specifically, the neurocranium width, tibial and femoral lengths resulted the most correlated radiographic measurements with the age. Based on them, a special equation was obtained and, in the future, it could become the best and more practical method to estimate the age in living newborn puppies. Obviously, further studies are required to verify the reliability of this equation, by increasing the number of the subjects enrolled. Additionally, it would be more advisable to find a similar equation based on the anatomical measurements, to estimate the neonatal age through an even more practical and fast technique compared to the radiographic examination.

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