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REPRODUCTION AND PERINATOLOGY IN COMPANION ANIMALS INVESTIGATED BY USE OF NONINVASIVE MATRICES

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“If you can see, look.
If you can look, observe.”

Blindness, J. Saramago

CONTENTS

ABSTRACT

Reproduction in companion animals is a topic of growing interest for scientific research, but knowledge about some aspects is still lacking. This is likely due to the need of collecting a large number of serial samples in order to perform longitudinal studies of the longer-lasting reproductive phases, which is now incompatible with the current concept of animal welfare, according to which any distress must be avoided, especially where pregnant and neonatal subjects are concerned. For these reasons, complying with the need of respecting animal welfare and aiming to study reproductive phases that are still incompletely explored in companion animals, the project lines of the present PhD thesis were focused on three crucial phases of reproduction: pregnancy and post-partum, perinatology and puberty. This was achieved by using matrices, like coat and the claws, and, to a lesser extent, fetal fluids obtained by noninvasive sampling. The sudden Covid-19 pandemic and the consequent lockdown rules have partially impaired the sample collection and laboratory analyses, but some interesting and satisfactory data were provided nonetheless. In the pregnancy and post-partum study, the results showed changes in the concentrations of cortisol (C) in coat from mating to 60 days post-partum, in line with what was reported for cats, suggesting that canine maternity could be considered as a challenge for the bitches, and dehydroepiandrosterone-sulfate DHEA(S) was also analyzed. About perinatology, the claws concentrations of C, DHEA(S), E2 and T tended to decrease from birth until 60 days of age, adding precious information about the perinatal physiology. In fetal fluids, leptin amniotic concentrations were higher in smaller-seized breeds, highlighting the role of breed body-size in affecting the intrauterine fetal metabolism. In the puberty study, in cats 17β-estradiol (E2) and testosterone (T) concentrations were assessed in coat and dewclaws. The concentrations of T in coat significantly differed between pubertal males and females and between gonadectomized males and females. The concentrations of T in coat were higher in pubertal than prepubertal male cats. In dogs, the analyses of the concentrations of C, DHEA(S), T and E2 in coat showed lower DHEA(S) concentration in pubertal than prepubertal and gonadectomized female dogs. In males, T concentrations in coat were higher in pubertal than prepubertal dogs, and C concentrations in coat were higher in pubertal than prepubertal and gonadectomized dogs, suggesting that pubertal status leads to a higher activation of the HPA axis in male dogs. The results shown in the present thesis evidenced the usefulness of these matrices for longitudinal and long-term hormonal studies of reproduction in dogs and cats, providing useful data about crucial reproductive phases and new interesting insights. Further investigations are needed to better understand some pending questions about the use of these matrices.

PREFACE

COMPANION ANIMAL REPRODUCTION AND PERINATOLOGY AT THE BEGINNING OF THE 3RD MILLENNIUM

Companion animals have always attracted and conveyed the interest of researchers in many disciplines, given their social, economic, and emotional relationship with the human being. After an initial major interest for horses and large animals, veterinary medicine shifted most of its concentration towards companion animals.

The primary aim of veterinary medicine in the past has been to limit canine and feline reproduction, in order to contain the excessive number of stray dogs and cats. Having achieved this objective with a satisfactory result, a new interest in reproduction performances has grown for companion animals, almost to the point of mimicking that for the bovine and equine species. In consequence of this, an in-depth scientific investigation of both the physiology and the pathology of reproduction is currently under way. The interest in veterinary small animal reproduction, previously centred on the female reproductive tract, has progressively grown to encompass all the aspects of male reproduction as well, and, finally, perinatology.

The constant impressive development of scientific knowledge allows for a continuous improvement in the management of reproduction and of the clinical approach to the recognized genital diseases. However, some reproductive phases, such as pregnancy and post-partum, perinatology and puberty, characterized by long-lasting physiologic changes, have yet not been fully analysed, leaving many questions still unanswered.

In response to the increased demand for a better knowledge of companion animal reproduction, the scientific community needs to guarantee a good progress in scientific research while abiding by animal welfare rules. The latter goal is achieved through many strategies, such as the "3R" rule and the development of noninvasive techniques of investigation allowing for a lesser distress of the patient.

A plethora of complex physiological phenomena are involved in reproduction. The hormonal cascade on which the control of reproduction is based is considered pivotal. For this reason, since the 70s, when most of the hormonal analyses were validated, the scientific interest has focused on the study of the main hormonal changes occurring during the reproductive processes. In the previous studies, hormonal concentrations were classically measured in blood or plasma by using venous blood collected by venipuncture. However, though blood sampling is still considered a useful procedure for research

purposes, it has recently been dubbed as invasive because it requires handling and perforating the skin of the patients, representing a source of stress for them, as well as potentially causing fear and/or pain. Indeed, fearful or overwrought animals can show additional restlessness and move during the procedure, making this difficult and potentially altering the results of the analyses.

Furthermore, the evaluation of hormonal changes that can occur during many reproductive processes often implies serial blood samplings, not complying with the need to guarantee the patients' welfare, especially considering that the variations could occur over a medium or long period of time, thus requiring multiple samplings.

Upset and stress can impair many physiological processes, and the negative effects of stress on reproduction in both humans and animals are well known.

Among the reproductive phases, pregnancy and post-partum are considered very sensitive to the negative effects of stress; this is especially true for animals, where even a mild upset can impair the pregnancy course or cause changes to the maternal behavior, hampering in turn the health of the offspring. The approach to the study of perinatology is even more delicate than the one used for pregnancy and post-partum. This physiological phase, considered as the interval of time elapsing from immediately before birth until the end of the neonatal period, is one of the most sensitive to stress, which can lead to serious consequences for the offspring. In companion animals, the study of perinatology (and neonatology) did not receive a great scientific interest until recently, and it is still currently one of the less investigated aspects in canine and feline reproduction.

This is due to a multitude of reasons. Firstly, until a few decades ago, it was considered normal, or at least irrelevant, to accept high perinatal losses in these polytocous species. This concept has now completely reversed, and high perinatal losses are considered unacceptable both from the ethical point of view and for the economic impact on dog and cat breeders.

A more technical reason for the lack of studies concerning canine and feline perinatology/neonatology could be that their small sizes make it almost impossible to collect classical matrices of study (such as blood). Performing blood withdrawals in these subjects is an invasive procedure, and could be harmful or even life-threatening, because of the limited total volume of circulating blood, the fragility of venous capillaries and the immaturity of the immune system. These risks prevent from collecting the suitable volume of blood needed for the analysis, let alone performing serial sampling.

Besides its "invasiveness", blood sampling carries another important issue which must be taken into consideration. In fact, blood provides only punctual information, very useful to measure instant changes, but requiring the serial repetition of samplings when studies are aimed to investigate long-

term hormonal changes, which are, as mentioned above, a typical feature of many reproductive phases, such as puberty, pregnancy and post-partum, perinatology, investigated in the present thesis. Indeed, the above described difficulty in performing serial blood samplings in companion animals, limiting the chances of assessing long-term hormonal changes, are one of the reasons why these phases of reproduction have not yet been completely investigated.

For all the above reasons, in the last years a growing interest has arisen in the development of new strategies of investigation, focused on the use of matrices alternative to blood, collectable without invasiveness, and providing the maximum amount of information with the minimum stress for the patient. However, more recently, attention has also been paid to the need of using matrices able to depict long-term changes, thus allowing for a reduction in the number of samplings and limiting the the distress to the patient.

The present thesis was therefore focused on the study of some reproductive phases in companion animals that are still not completely understood, such as pregnancy and post-partum, perinatology and puberty (all of which characterized by long-term hormonal changes) by using new research matrices, collectable without invasiveness. The final goal was to provide new scientific basic knowledge about companion animal reproduction, and also possible useful information for a more specialized practical management and clinical assistance to canine and feline reproduction.

The thesis summarized a 3-year PhD project involving 3 parallel study lines, focused respectively on pregnancy and post-partum, perinatology and puberty in companion animals. These 3 reproductive phases are characterized by very different time of observation: about 120 days for the study of pregnancy and post-partum; about 60 days for the study of perinatology, and about 4-12 months for the study of puberty, that influence also the timing of samples and data collection. In fact, although all the 3 study lines started at the 1st year of the PhD course, the finalization of samples collection and of the data obtained was expected to be different among the 3 study lines, with reasonable conclusion at the end of the PhD course.

Unfortunately, the sudden Covid-19 pandemic emergency at the beginning of March 2020 and the consequent lockdown, prevented the conclusion of part of the studies, especially for the 2 project lines characterized by the longest time of observation: pregnancy and post-partum, and puberty. In fact, access to breeding facilities and to the veterinary faculty were forbidden for more than two months impeding the samples and data collection from those subjects already recruited for the studies, other than preventing the enrollment of new subjects. Therefore, some on-field data were not useful because not completed or interrupted. Moreover, the emergency restrictions involved also the laboratory where

the analyses were scheduled, and reopening was allowed with a reduced number of working people, so that also the planned analyses had to be delated and strongly limited.

Therefore, at the time of the writing of the present thesis, satisfactory data were collected from the study line about perinatology, whilst preliminary results were available about the study lines related to pregnancy and post-partum, and to puberty.

INTRODUCTION

Hints about the study of reproduction

When reproduction is concerned, some considerations must be advanced before deepening this interesting field of research. The reproductive activity permeates the everyday life of each type of living being, and it is directly connected to the survival of a species. The diverse compartments of an organism, once considered as separated entities, are now studied not only regarding their intrinsic function and physiology, but also in relation to the other physiologic systems. This allows to better understand the multitude of interplays among the organs, how one is able to influence the others, and vice versa (Marceau et al., 2015). About reproduction, it is now clear that its functionality is directly interrelated with the mechanism of stress and the development of the organism, as showed in literature (Dubey and Plant, 1985; Battaglia et al., 1998; Viau, 2002; Valsamakis et al., 2019). This thesis will consider pregnancy and post-partum, perinatology and puberty, three phases in which the maturity and the ability to cope with external and internal stressors is mandatory to overcome without any residual problem those periods. For these reasons, before starting to explore the physiology and the dynamics of the three phases aim of the thesis, it could be helpful to understand how those two functions, reproduction and stress, are regulated in the organism and how they influence each other.

Hypothalamic-Pituitary-Adrenal axis and Hypothalamic-Pituitary-Gonadal axis in reproduction

The Hypothalamic-Pituitary-Adrenal axis

During the course of life, every living being is subjected to a wide range of stimuli, also reported as "stressors", that can have negative effects, sometimes even to the point of endangering the life of an organism. The adaptive responses of the body to external stressors is a key-system for the survival of a subject, because it allows the return to a condition of homeostasis (Goel et al., 2014). When adaptiveness to stressors is concerned, the Hypothalamic-Pituitary-Adrenal (HPA) axis is one of the main regulating systems of the body in humans and animals (Goel et al., 2014). Some of its actors are based in the central nervous system and some are located at the peripheral nervous system (Valsamakis et al., 2019).

The activation of this axis results, in turn, in a cascade of events. As its name suggests, the first step takes place in the hypothalamus, in particular in the paraventricular nucleus, where the secretion of Corticotropin-Releasing-Hormone (CRH) and Arginine Vasopressin (AVP) are produced (Goel et al., 2014). After their arrival in the hypophysis, these two hormones synergistically stimulate the anterior part of this gland for the secretion and release of Adrenocorticotropin Hormone (ACTH) (Viau, 2002; Goel et al., 2014). Eventually, ACTH stimulates the adrenal cortex, from which the synthesis and release of glucocorticoids starts (Goel et al., 2014). In particular, ACTH elicits the conversion of cholesterol to pregnenolone, that is a precursor for cortisol (C) and Dehydroepiandrosterone (DHEA) (Whitham et al., 2020). The sulfated form of DHEA, DHEA sulfate (DHEA-S), is reported to be more stable than DHEA and it is the result of the hydroxysteroid sulfotransferase activity, more commonly indicated as DHEA sulfotransferase (Maninger et al., 2009). On the contrary, the reconversion of DHEA-S in DHEA is possible through the activity of steroid sulfatase (Maninger et al., 2009). Cortisol, commonly known as "the hormone of stress", can have a huge spectrum of action on the different organs. As a first step, it gives rise to the production of glucose, needed to cope with the temporary stressor that have activated the axis (Lee et al., 2012; Kamin and Kertes, 2017). In the meantime, and especially when this hormonal pattern is increased in a long-term manner, it momentarily exerts an inhibiting action on other functions, like growth and reproduction (Kamin and Kertes, 2017). This action can be interpreted as a natural response for which the organism selects the activities that are

essential to overcome the instantaneous stressor and survive, redirecting the available glucose to the essential compartments.

Other than C, also DHEA seems to follow a circadian pattern of secretion, with highest concentrations in the morning reported in humans (Kroboth et al., 1999; Collomp et al., 2014; Marceau et al., 2015). However, in golden hamsters, higher concentrations of DHEA and DHEA-S were reported in the evening compared to the morning (Pieper and Lobocki, 2000). The C secreted is then able to exert a negative feedback action on the axis: when plasma glucocorticoid levels are high, the axis is inhibited (Viau, 2002). On the contrary, in literature there is no evidence of this mechanism of inhibition via feedback system by DHEA-S on the HPA axis (Leowattana, 2004; Kamin and Kertes, 2017). This regulatory feedback, however, is of greatest importance in avoiding the disrupting of this axis: in fact, when the stressor has ceased, the axis stops to stimulate the production of C (Kamin and Kertes, 2017). The stressor that activates this axis can be real or only perceived (Goel et al., 2014; Valsamakis et al., 2019). Exhausting exercise, diseases, psychiatric disorders are among the most common sources of stress reported in humans (Rivier and Rivest, 1991). In horses, transportation, pain and even social stressors are common causes of increased production of glucocorticoid (Merl et al., 2000; Aurich and Aurich, 2008; Schmidt et al., 2010; Deichsel et al., 2015; Nagel et al., 2017). Notably, even horses accustomed to transportation exhibit pattern of increased C during and after this type of stressor (Deichsel et al., 2015). HPA axis has effects on a multitude of regulatory systems, as immunity, cognition, behavioral system, but also cardiovascular system, with genomic and nongenomic effects (Rosado et al., 2010; Lee et al., 2012; Kamin and Kertes, 2017). Other than that, there is now a clear evidence of its important influence also on reproductive activity and efficiency, with the eventual inhibition of the production of reproductive hormones (Valsamakis et al., 2019; Whitham et al., 2020). This concern has important consequences in reproduction, with different types of clinical implications (Valsamakis et al., 2019). When the physiology of this axis is concerned, some peculiar differences between male and female subjects were reported in literature, especially about human species. For instance, production of DHEA and DHEA-S occurs only in the adrenal glands in women, whilst in man, 10-25% of the DHEA and 5% of the total DHEA-S are produced by the gonads, i.e. the testes (Bird et al., 1984; Leowattana, 2004). In golden hamsters, gonadectomy did not influence significatively the production of DHEA and DHEA-S, on the other hand the removal of the adrenal glands created a conspicuous decrease of DHEA-S (Pieper and Lobocki, 2000). In the same work from Pieper and Lobocki (2000), moreover, a decrease of both DHEA and DHEA-S concentrations related to aging was seen, in accordance with what is reported in humans (Birkenhäger-Gillesse et al., 1994; Kroboth et al.,

1999). Whilst the details about the functionality of this axis have been described, some information are lacking about the influence of the sexual steroid hormones on this axis (Viau, 2002).

The Hypothalamic-Pituitary-Gonadal axis

The main regulating system of reproduction is represented by the hypothalamic-pituitary-gonadal (HPG) axis and its interaction with the body and the environment. The first actor of this complicated network is the hypothalamus, a little region of the brain, that, thanks to the interplay with several types of neurotransmitters, opiates, melatonin (the secretion of which is associated with the number of daylight hours), and some neuronal and hormonal feedback systems, secretes the gonadotropin-releasing-hormone (GnRH). Once secreted by the neurons of this region, GnRH is released from the nerve termination in the median eminence. Thanks to the hypophyseal portal system, also reported as "pituitary-portal system", GnRH is carried to the anterior part of the pituitary gland, where it stimulates the secretion and release of the gonadotropin hormones, i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH). Those hormones have a direct influence on the final actors, that are the gonads (England, 2013a; England, 2013b). In the female, this process drives folliculogenesis, ovulation, maintenance of corpus luteum and synthesis of ovarian hormones, above all estrogens and progesterone (England, 2013a; Plant, 2015). In males, this axis guides the spermatogenesis and the secretion of testosterone (T) (Feldman and Nelson, 1992; England, 2013b; Plant, 2015; Zeleznik and Plant, 2015; Kaprara and Huhtaniemi, 2018). Circulating androgens (and estrogens) exert a negative feedback on this pathway, exerting a balancing feedback effect (England, 2013b; Dart, 2014; Kaprara and Huhtaniemi, 2018). Despite the common negative feedback system, the changes of FSH and LH do not always run in parallel. For this reason, the existence of an inhibitory factor able to exert its action only on the production of FSH was proposed with the name of "inhibin", a gonadal hormone (England, 2013b; Kaprara and Huhtaniemi, 2018). A study from Majumdar and coworkers (1995) on rhesus monkey (*Macaca mulatta*) reported that the action of inhibin is exercised at the level of the pituitary gland, regulating directly at this level the secretion of FSH (Majumdar et al. 1995). On the contrary, "activin" was proposed as a stimulatory factor that increases the production of FSH and are produced directly in the gonads by the Sertoli cells (England, 2013b). In males, systemic testosterone is produced by the Leydig cells of the testes and have multiple functions, among which a fundamental role in the spermatogenesis (Chłopik and Wysokińska, 2020). In fact, the functionality of Sertoli cells is directly

dependent on the concentration of T, considering also that higher concentrations are found in the testicular parenchyma than in the peripheral circulation (England, 2013b). During canine proestrus, the development of the follicular structures is stimulated by FSH and LH, that will in turn start to produce estrogens from the granulosa cells (England, 2013a). In a work on ewes, Goding and coworkers reported that the 17β-estradiol (E2) produced was able to exert a positive feedback on the HPG axis, in particular stimulating an increase in LH (Goding et al., 1969).

Disorders at the pituitary-gonadal level of this axis can, in turn, result in androgen deficiency in males and androgen excess or deficiency in females (Dart, 2014). These hormones not only act on specific and targeted tissues, but have many other influences, like action on other tissues, on behavioral patterns, and, in the end, concurring to the physiologic maintenance of the individual homeostasis (Viau, 2002). In humans, this axis is reported to go towards an activation during midgestation in the fetus, followed by a gradual turning off at the end of pregnancy (Lanciotti et al., 2018). The cause for this deactivation seems to lie in a negative feedback of the placental hormones on the hypothalamus of the fetus (Lanciotti et al., 2018).

As HPA axis is known to exert a lot of effects permeating the physiology and functionality of an organism, the same concept is now thought to be applicable on HPG axis, as its final products, sexual steroid hormones, seems to mediate diverse effect on the living beings. "Outside the realms of the gonadal tissues, many tissue-specific effects of androgens, are very subtle and overlooked" stated Dart in 2014. For instance, in humans, olfactory bulb (a region of the brain) seems to be diversely shaped between men and women, and cardiovascular disorders are reported to display sexually dimorphic differences (Kalin and Zumoff, 1990; Dart, 2014). The influence of the sexual steroid hormones is reported to start in the very early stages of life, i.e. the intrauterine development (Sathishkumar et al., 2011). Whilst a lot of studies focused on HPA axis, less is known about the HPG axis until now. The renewed interest about this axis and its role has opened new questions, and further research is needed. Furthermore, given the peculiar differences in the pattern and regulation of this axis among different species reported above, a specie-specific approach to this matter of study is recommended.

Effects of the interactions between HPA and HPG axes on reproduction

One of most representative cases of the reciprocal influence between HPA and HPG axes is the inhibition of reproductive axis, included the secretion of sexual hormones, played by the stress (Viau,

2002). This statement is particularly effective when chronic stress is concerned, whilst it is less clear the role of an acute stressor (Tilbrook et al., 2000; Aurich and Aurich, 2008). In women, a condition called "Functional hypothalamic amenorrhea" is reported, in which a chronic anovulation is present without specific organic causes (Valsamakis et al., 2019). Although no specific reasons can be identified, this physical condition is often attributable to slimming and general stressors in life (Valsamakis et al., 2019). The stress is reported to be responsible for the functional reduction of the GnRH release (Gordon et al., 2017). When GnRH reduction is consistent, the LH and FSH activity is insufficient to maintain the ovulatory activity and folliculogenesis (Gordon et al., 2017). In fact, in these women, a decrease in frequency and an increase in the interval-time among the LH pulses is seen, and this, in turn, creates a condition of hypoestrogenism and then amenorrhea (Berga et al., 1989). It is furthermore reported that corticosteroids inhibit gonadotropin secretion, but the exact mechanism through which this happens is not completely clear, even if a role of opioids and catecholaminergic systems targeted at the higher brain centers and hypothalamus is supposed (Brann and Mahesh, 1991). If the reports about chronic stress are almost in accordance about the suppression of the HPG axis, about acute stress the published studies differ in their results (Tilbrook et al., 2000; Valsamakis et al., 2019). Indeed, variable effects from stress are reported in rodents and other animals (Brann and Mahesh, 1991). When 4 hours of isolation and restraint were used as a source of stress in gonadectomized rams and ewes, the basal secretion of LH decreased, with different patterns of amplitude or frequency of the pulses, depending on the experimental group (Tilbrook et al., 1999). The same type of stressor was applied for 6 hours in rhesus monkeys, creating an inhibitory effect on the secretion on LH in both males and females (Norman and Smith, 1992; Norman et al., 1994). In a work from Matteri and colleagues (1984), a restraint of 3 hours was sufficient to induce a decline in the basal secretion of LH in rams. Other than this, sexually dimorphic differences in the HPA response to stressor are described (Goel et al., 2014).

Another mechanism through which these two axes are connected is the elevation or depletion of glycaemia. It is well known, indeed, that stress can act through the HPA axis to stimulate the gluconeogenesis and glycogenolysis that, in the end, increase the levels of circulating glucose (Marik and Bellomo, 2013). In 1990, Clarke and colleagues experimentally injected intravenously 100 UI insulin to ovariectomized ewes, and reported a decrease not only in glycaemia but also in the secretion of LH. However, if glucose was administered intravenously in addition, the effect of insulin on the secretion of LH disappeared. This latter result was, for the authors, the proof of the mediated effect of insulin on the pattern of LH secretion by the neuroglycopenia (Clarke et al., 1990).

Other than this, an additional way through which the HPA axis is activated and can influence the reproductive axis is an inflammatory challenge (Battaglia et al., 1998). For understanding the extent of this influence, Battaglia and colleagues (1998) injected 400 ng/kg of endotoxins intravenously in ewes. After this stimulus, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) hormone concentrations increased in the hypophyseal portal blood. Contextually, GnRH and LH pulsatile secretion were suppressed, whereas cortisol and progesterone secretion were increased. This is an additional confirmation about the influence between these two axes (Battaglia et al., 1998).

On the other hand, some reports of a stimulatory effect played by acute stressors on the HPG axis exist. After a study on boars injected with cortisol, it was reported that elevation in plasma concentrations of C could amplify the secretion of LH and, consequently, of T (Liptrap and Raesie, 1983). The mechanism through which this happens could be addressed to the enhanced responsiveness of the anterior pituitary gland to GnRH (Liptrap and Raesie, 1983). Other than this, a short increase of plasma T concentrations subsequent an injection of dexamethasone was reported in young bulls (Thibier and Rolland, 1976). However, after the injection of dexamethasone, the authors reported a suppression of LH pulsatile secretion. Besides this, the little increase of T levels rapidly decreased after 4 hours from the injection (Thibier and Rolland, 1976). The results obtained suggested that C directly inhibits the secretion of LH that, in turn, results in lower concentrations of T (Thibier and Rolland, 1976). In prepubertal gilts, on the other hand, cortisol injection in bloodstream created an elevation in endogenous LH levels (Pearce et al., 1988). Besides this, in the same species, animal transportation is reported to stimulate the attainment of puberty (Hughes, 1982).

In a work from Deichsel and coworkers (2015), 13 Shetland stallions of proven fertility were exposed to transportation or to serial injections of ACTH (every four hours). The aim was to evoke a stress response either in a practical way, that is transportation, or in a pharmacological way, that are the ACTH injections. Both ways led to an increase in salivary C concentrations (Deichsel et al., 2015). However, this increase did not result in a suppression of the secretion of T and on spermatogenesis. In fact, plasmatic concentrations of T were not affected during the experimental trial, as well as the semen parameters (Deichsel et al., 2015). It seems that, being horse a prey and given that increases of C after stressful stimuli are often seen in this species (Merl et al., 2000; Gordon et al., 2007; Aurich and Aurich, 2008; Schmidt et al., 2010; Deichsel et al., 2015), protection of the reproductive function is a natural system achieved to guarantee reproductive efficiency and then the survival of the species (Deichsel et al., 2015). Indeed, also in mares undergoing transportation for long road trips an increased C release was seen in serum concentrations, but, similarly to males, no depletion of the estrus activity or

irregularity of the estrus cycle were seen. On the contrary, higher concentrations of LH were seen in transported than in non-transported mares (Baucus et al., 1990a quoted by Deichsel et al., 2015). Even when pregnant mares are involved, no increase in the embryonic mortality was seen (Baucus et al., 1990b quoted by Deichsel et al., 2015).

In humans, however, when the stressor is a vigorous physical exercise of short duration, the elevation of T seems to be due to a less performing metabolic clearance of this hormone than a real increase in its production, that is thought to be attributable to a decrease in the hepatic bloodflow (Rowell, 1974; Rivier and Rivest, 1991). However, as reported by Rivier and Rivest (1991), it is important to notice that serum concentrations of LH and testosterone are not always correlated. Thus, one should be prudent when interpreting results about these hormones, unless they represent the result of longitudinal assays during stress (Rivier and Rivest, 1991).

As anticipated above, a lot of studies are in accordance in establishing a suppressive role of stress on reproductive efficiency, in both human and animal species (Valsamakis et al., 2019; Tilbrook et al., 2000). A long period of stress seems to be necessary for inducing a suppression of the HPG axis, as revealed by a work on gonadectomized rhesus monkeys in which the suppression was obtained only after 21-28 days of treatment with cortisol (Dubey and Plant, 1985; Tilbrook et al., 2000). Also in humans, chronically high plasma C concentrations are associated with impaired reproductive performances (Tilbrook et al., 2000). Experimentally daily exposure to mild stress was not able to induce modifications in the total length of the estrus cycle and the length of each phase in mice (Wagenmaker and Moenter, 2017). When the stimulus was narrowed to a single psychosocial stressor, modifications on the LH surge were seen (Wagenmaker and Moenter, 2017). This latter effect seems to be due to a disrupted production of estradiol that, in turns, is not able to exert its positive feedback effects on the production of LH (Wagenmaker and Moenter, 2017). Thus far, however, these results clearly indicate a role of the stress in modulating the HPG axis and, in turn, the reproductive efficiency of a subject. Already in 1991, Rivier and Rivest reported the necessity to precisely address what were the sites in which these mechanisms of modulation take place, and, even if in the meanwhile new discoveries and a lot of new information were provided, further research is needed to clarify at what levels and with what mechanisms sex steroids interact with the brain (Handa and Weiser, 2014). Indeed, most of the reports about the effects of sexual steroid hormones on HPA axis activity are reported to be insufficient and fragmentary (Dallman et al., 2002).

In some studies, the depletion of LH after the cortisol treatments was not accompanied by a repression of the LH surge after the release of GnRH (Dubey and Plant, 1985; Juniewicz et al., 1987). This led to

the conclusion that the site of action of the glucocorticoid is the hypothalamus (Tilbrook et al., 2000). Nevertheless, a peripheral action is also reported, and every component of the HPA axis result to be able to have an influence on the HPG axis at multiple levels, as reported below (Figure 1) (Rivier and Rivest, 1991; Valsamakis et al., 2019). In ovariectomized Sprague-Dawley rats, glucocorticoids can suppress the HPG axis at every level; hypothalamic, pituitary, but also ovarian and uterine functions seem to be affected by C (Rabin et al., 1990). In 2000, the existence of an inhibitory peptide that can act on the anterior pituitary impeding the release of GnRH was reported (Tsutsui et al., 2000). Albeit this study was performed on in vitro cultured anterior pituitaries of quails, representing a preliminary step, it is clear how complicated and dense is the regulation of this axis.

A role of these two axes can be presumed also when maternal behavior is concerned, given the major role played by E2, P4 and C in shaping the parental care (Gonzalez-Mariscal and Melo, 2013; Lévy, 2016). Notably, E2 is reported to be essential for maternal care, whilst the decline of P4 before parturition concurs to the onset of maternal behavior with parturition (Lévy, 2016). About the HPA axis, in sheep a role of this axis in the display of maternal behavior was presumed, whilst in rats a correlation between "licking behavior" as a part of the maternal cares and the concentrations of corticosterone is reported (Lévy, 2016). In humans, HPA axis is reported to play a role in the mother-infant bonding (Mastorakos and Ilias, 2000).

Another interesting influence of "reproductive" axis on "stress" axis was described in rats. A severe decrease in the responsiveness of the HPA axis to external stressors, indeed, was reported in pregnant and lactating rats (Neumann, 2001). This particular process of desensitization seems to be mediated by the action of oxytocin and prolactin, another time confirming how complex this interplay is (Neumann, 2001).

Lastly, also in neuroendocrinology some evidences that a remarkable number of neuropsychiatric disorders show huge differences based on the gender. Given that those neuropsychiatric diseases have a pathogenesis of HPA axis dysregulation and given these gender differences, a modulatory role of the sexual steroid hormones on this axis can be presumed (Handa and Weiser, 2014).

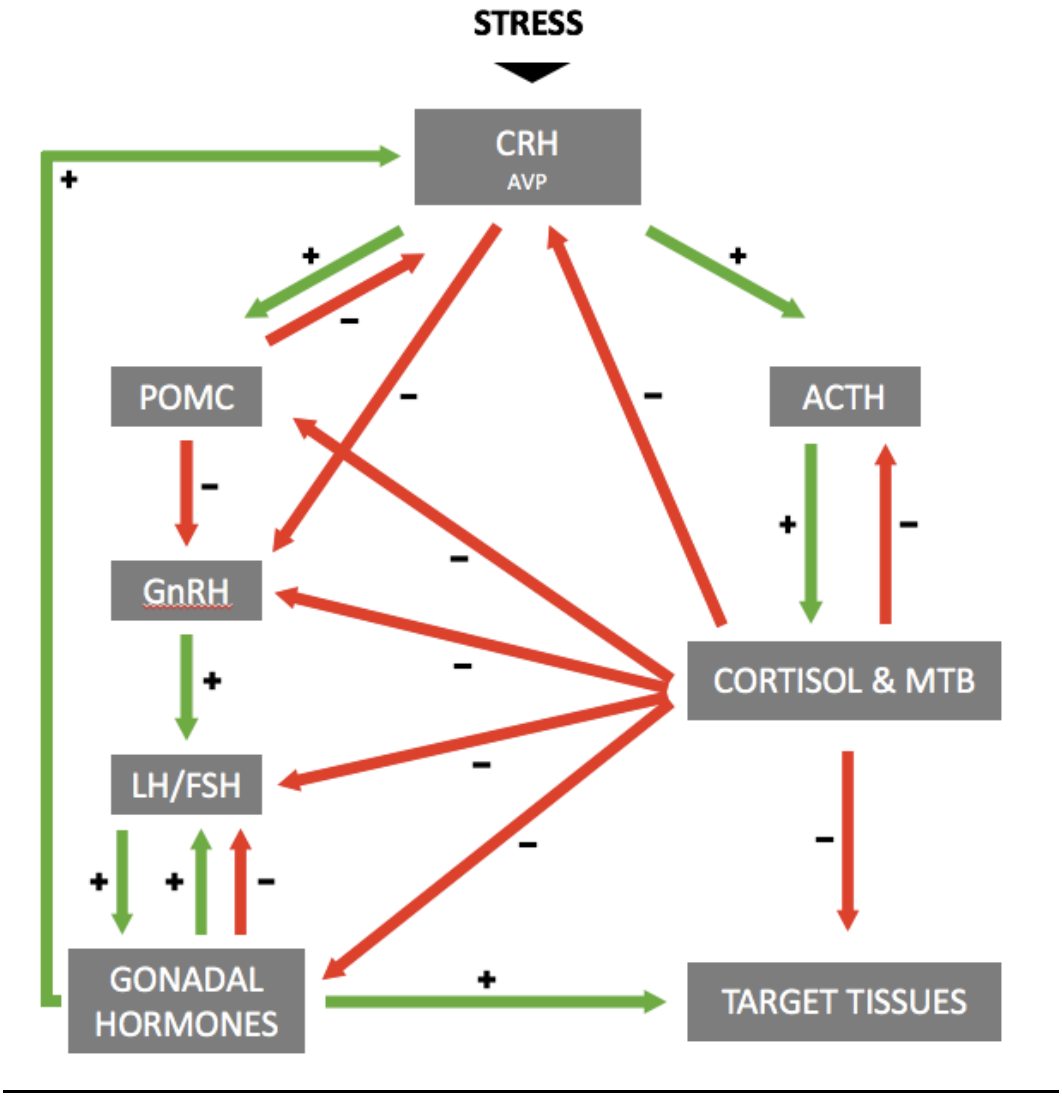

Figure 1. Exemplified scheme of the multiple levels of interactions between HPG axis, on the left, and HPG axis, on the right, when there is a stressor. Green arrows with "+" symbol indicate stimulatory activity, red arrows with "-" symbol indicate inhibitory activity. **CRH**: Corticotropin Releasing Hormone / **AVP**: Arginine Vasopressin / **POMC**: Pro-opiomelanocortin / **GnRH**: Gonadotropin releasing Hormone / **LH**: Luteotropic Hormone / **FSH**: Follicle-stimulating Hormone / **ACTH**: Adrenocorticotropic Hormone / **MTB**: metabolites. Adapted from the images of Mastorakos and Ilias, 2000; Tilbrook et al., 2000; Valsamakis et al., 2019.

Reproductive phases aim of the study

Pregnancy and post-partum in companion animals

Pregnancy is one of the most peculiar and interesting phases of reproduction. During this period, a new hormonal pattern is set: this is essential not only for the establishing of the pregnancy itself, but also for the correct course of it, for the health of both the mother and the fetus(es). Although dogs and cats are both carnivores, some specie-specific peculiarities of the physiological hormonal pattern exist.

For what regards dogs, some peculiarities start already at heat. Firstly, dog is a nonseasonal species with an interestrus interval of approximately 5 to 12 months (Gudermuth et al., 1998). In addition, a specie-specific characteristic of the canine ovarian cycle is the pre-ovulatory rise of progesterone serum concentrations, caused by the pre-ovulatory luteinization of some follicles that create an elevation of circulating progesterone concentrations already from the proestrus phase (Edqvist et al., 1975; Verstegen-Onclin and Verstegen, 2008).

In a work from Edqvist and colleagues (1975), the circulating serum concentrations of progesterone were under or around 1 ng/ml before the onset of proestrus (now recognized as "basal" levels), eventually reaching 10 ng/ml at the onset of estrus. A more recent work on 1300 bitches reported a mean level of 4.8 ± 0.9 ng/ml and 7.2 ± 1.3 ng/ml at 2 and 3 days after LH surge, respectively, that is the time around which ovulation is reported to occur (Hollinshead and Hanlon, 2019). After the onset of estrus, the concentration of progesterone gradually increases and peaks at 30 - 50 ng/ml (Edqvist et al., 1975). Unfortunately, huge individual differences in the concentrations of progesterone are observed, and similar endrocrine profiles are observed between pregnant and non-pregnant bitches, making this hormone not useful for the diagnosis of pregnancy (Edqvist et al., 1975; England, 2013a). In fact, unlike other domestic animals, the functionality of the corpus luteum is independent from the pregnancy status in dog, so that the hormonal production is very similar in pregnant and non-pregnant bitches (Concannon et al., 1978; Hoffmann et al., 1992; Papa and Hoffmann, 2011). The serum P4 levels during the diestrus, indeed, either in pregnant or non-pregnant bitches, can increase until 15 - 80 ng/ml and then decrease in proximity to the end of this phase (Davidson and Feldman, 2015). The CL reach their highest hormonal production from 25^{th} - 35^{th} day, with a subsequent decrease in P4 secretion (Papa and Hoffmann, 2011). The only difference is seen towards the end of the luteal phase: indeed,

because of the pre-partum hormonal decrease, pregnant animals returns earlier and in a sharper way to the basal concentration of progesterone (P4) (Concannon et al., 1978; Papa and Hoffmann, 2011).

On the other hand, what joins dogs with other species is the preovulatory LH surge, some days after the estrogen elevation. This increase, that happens around 2 days before ovulation, is characterized by a pulsatile manner (Plant, 2005). Another hormone that increases in the proestrus phase is 17β-estradiol (E2), with a rise followed by a decrease, that is parallel to the elevation of progesterone (England, 2013a). E2 is reported to increase from 5 pg/ml to values of 40-90 pg/ml in circulating blood samples, causing the proliferation and modification of vaginal epithelium (Concannon, 2009). The onset of estrus is parallel with the decrease of E2 (Concannon, 2009). Other than this hormonal pattern, proestrus phase is characterized, in general, by the female refusing the mating approach of the male (England, 2013a). The contemporary elevation of P4 and decrease of E2 is thought to be necessary for the expression of the estrus behavior, which includes the acceptance of mating (Concannon, 2009; England, 2013a). Canine females have multiple spontaneous ovulations, with ovarian follicles ovulating 48-60 hours after the LH surge (England, 2013a). In some cases, however, ovulation of follicles even after 96 hours after the LH peak are described (England, 2013a). The proestrus phase is variable in its duration, reported with a mean of 9 days, even if ovulation can occur 5-25 days after the beginning of proestrus (Concannon, 2011; England, 2013a). Some variability related to breed have been described: in German Shepherd, short estrus cycles have been reported to be associated with compromised fertility (Günzel-Apel et al., 2016). However, no significant differences were reported between the two different body-size breeds, such as the Bernese Mountain Dog and Cavalier King Charles Spaniel, in relation to P4 concentrations during estrus cycle (Thejll Kirchhoff and Goericke-Pesch, 2016).

Even if different from the canine peculiarities, also cats show some particular characteristics in their reproductive physiology: they are, indeed, seasonal poliestral breeders in which the ovulation does not occur in a spontaneous way but requires the coitus, and normally appears 24 - 36 hours after copulation (Concannon et al., 1980; Wildt et al., 1981; Tsutsui and Stabenfeldt, 1993; Siemieniuch et al., 2012). Based on the report of Foster and Hisaw of 1935, the reproductive season of female cats goes from January until September, but this must be related to the latitude and local climate (Hurni, 1981). Some authors reported two peak seasons of activity, from January through March and from May through June (Verhage et al., 1976). During the reproductive season, estrus occurs repeatedly every 2-3 weeks (Tsutsui and Stabenfeldt, 1993). In a work from Schmidt and colleagues, the average duration of every estrus cycle (including only the proestrus and estrus phases) is reported to be around 5.4 ± 0.4 day; in

addition to that, they found that the post-partum estrus was significantly shorter, 3.8 ± 0.5 day (Schmidt et al., 1983). A prompt growth of corpora lutea (CL) producing P4 is described in both pregnant and pseudopregnant cats (Siemieniuch et al., 2012). In cats, ovulation is stimulated by the matings, and the luteal phase can follow the estrus independently from the fact that fertilization occurs (England, 2013a). In non-pregnant cat, luteal phase lasts around 24 - 25 days, whilst in the pregnant cat it lasts approximately 65 days (England, 2013a).

When ovulation without fertilization occurs, the CL reach the highest production of progesterone in the first 10 - 15 days after ovulation and then decline, reaching the basal values around the 30^{th} - 35^{th} day of pregnancy (Tsutsui and Stabenfeldt, 1993). A work from Wildt and colleagues in 1981 investigated the estrus phase of female cats that were stimulated by mounts with vasectomized male cats. They reported an increase in E2 in the first three days of the feline estrus, with individual variations among the different subjects. Serum E2 values around 20 pg/ml are reported to be associated with sexual receptivity and follicular activity (Wildt et al., 1981). After the sixth day of estrus, the E2 serum concentrations declined under the 20 pg/ml, and, after this, concentrations were around 13 and 24 pg/ml during the first 35 days of the interestrus phase (Wildt et al., 1981). The circulating progesterone (P4) peak rose instead from the day 4 (to 14) after ovulation, with a concentration between 30.9 - 87.8 ng/ml from the day 9 to 23, variably distributed in the population enrolled in the study (Wildt et al., 1981). The authors, however, reported different amplitude of fluctuations of progesterone concentrations in circulating blood, in particular in the middle of the luteal phase. After this phase, progesterone drops and no more elevations were reported.

When pregnancy is established, a new hormonal pattern is set, both in dogs and cats. Progesterone is the fundamental hormone for the establishment and maintenance of pregnancy, and in the dog it is totally of luteal origin (Concannon, 2011). Even if, as mentioned above, similar hormonal patterns were described between pregnant and non-pregnant bitches, it must be noted that the metabolism and hepatic clearance of progesterone is significantly higher in the pregnant subjects, as highlighted by the different progesterone concentration in fecal samples from pregnant bitches after day 25 of pregnancy (Gudermuth et al., 1998; Verstegen-Onclin and Verstegen, 2008; Concannon, 2011). Besides this, however, the remarkable increase in plasmatic volume contribute to the dilution of the real titers of progesterone concentrations, so that in the end the concentrations of circulating progesterone in pregnant or non-pregnant bitches is similar (England, 2013a). However, in the bitch, progesterone is not the only hormone important for the maintenance of pregnancy. Indeed, LH and prolactin have a luteotropic action (Papa and Hoffmann, 2011). Higher concentrations of prolactin after day 25 tend to

confirm its role in sustaining the progesterone secretion (Verstegen-Onclin and Verstegen, 2008). In particular, prolactin is thought to be the main pituitary hormone that supports the production of steroid hormones from the CL (Verstegen-Onclin and Verstegen, 2008). Relaxin is another crucial hormone, secreted entirely from the placental tissue from mid-pregnancy; it is detectable from 25 days after ovulation (21-24 days after the LH surge), has a peak around the 50th day of pregnancy, and continue to be detectable until the end of pregnancy (Klonisch et al., 1999; Concannon et al., 2001; Verstegen-Onclin and Verstegen, 2008; England, 2013a). In canine species, the serum concentrations of relaxin are reported to be around 10 μg/ml during pregnancy (Steinetz et al., 1996).

Around day 40 of pregnancy, canine CL are reported to be able to synthesize E2, and a higher number of cells able to perform this hormonal pathway is described in the late stage of pregnancy (Nishiyama et al., 1999). However, the concentrations of E2 do not seem to differ between pregnant and not pregnant bitches, and, in both cases, it tends to constantly increase during the luteal phase (England, 2013a).

At the end of pregnancy, luteolysis occurs. From the physiological point of view, at-term labor and the starting of the process of parturition can be more attributable to the loss of the inhibitory effects of pregnancy on myometrium than an active process involving uterine stimulants (López Bernal et al., 1995; Norwitz et al., 1999; Johnson, 2008). Among the factors that contribute to uterine quiescence during canine pregnancy there are progesterone and relaxin, whilst prostaglandin and oxytocin are known to stimulate uterine activity ("uterotonic" action) (Johnson, 2008). The process is indeed started by a feto-placental mechanism, which creates a uterine and placental cascade that in turn produces prostaglandin F2α (PGF2α) and, after that, of 13,14-dihydro-15-keto-prostaglandin F2α, a prostaglandin metabolite (PGFM) (Concannon, 2009).

Prostaglandin F2α is reported to have important effects like relaxation of the birth canal, softening of the cervix and stimulation of uterine contractility, together with the endocrine and paracrine action of oxytocin (Verstegen-Onclin and Verstegen, 2008). After the release of these prostaglandins, serum concentration of P4 starts to decrease, arriving to reach <1 ng/ml during the 24 hours prior to the start of labor at the physiologic term of pregnancy, with the exception of the singleton pregnancy, in which higher levels of progesterone (>2 ng/ml) could be maintained during this final phase (Johnson, 2008; Concannon, 2009). In periparturient bitches, levels of circulating P4 decreased significantly 24 hours before whelping, reaching 9.3 ± 5.6 nmol/l (corresponding to about 2.6 ± 1.6 ng/ml) and continuing to decrease up to parturition, when the reported mean was 3.2 ± 1.2 nmol/l (corresponding to about 0.91 ± 0.3 ng/ml) (Veronesi et al., 2002). In Labrador bitches, circulating progesterone concentrations equal or

under 6 nmol/l (around 2 ng/ml) were found to be able to start the labor (Chakraborty, 1987). According to reports, this mechanism of PGF2α/PGFM production and progesterone decrease is hastened by a "cascade-like manner", because as P4 declines it loses its inhibitory effects on the PGF release (Concannon, 2009; Kowalewski et al., 2010; Nowak et al., 2019). With this regard, an important role in initiating this cascade is ruled by the fetus, that produces cortisol (C) from its adrenal glands after the maturation of the hypothalamic-pituitary-adrenal (HPA) axis (Challis et al., 2001; Veronesi et al., 2002). Its role could be exerted not only through the upregulation of the enzyme PGH-synthase-2, but also through the suppression of prostaglandin catabolism (Challis et al., 2001; Concannon, 2009). In a work from Veronesi and colleagues (2002), a positive correlation between C and 15-ketodihydro-PGF2α and a negative correlation between 15-ketodihydro-PGF2α and progesterone was observed in periparturient bitches. Given this active role of the fetus just described, it is easy to infer why any disturbance to the fetus during its intrauterine life is dangerous, and could prematurely trigger the mechanism of labor (Norwitz et al., 1999).

In dogs, pregnancy duration is around 2 months, more precisely 65 ± 1 days after LH peak and 63 ± 1 days after ovulation (Concannon et al., 1983; Kutzler et al., 2003). In cats, the pregnant luteinic phase lasts a mean of 65 days, and the pregnancy length goes from 52 to 74 days from the mating to the parturition, considering the difficulty in precisely estimating the time of ovulation (Levy and England, 2013). Indeed, cats are an ovulation-induced species and a variable number of matings are needed in order to stimulate the LH peak: other than that, there is no pre-ovulatory luteinization of the follicular cells of the granulosa, so that progesterone concentrations increase only after the occurrence of the ovulation induced by coitus (Johnston et al., 2001a; Beccaglia et al., 2016). Opposite to dogs, furthermore, in cats pregnant and non-pregnant luteinic phases have different durations: in the former condition, it has a reported length of around 65 days, in the latter it lasts only 30-40 days, in which, however, no estrus signs are showed (Levy and England, 2013). Another important difference lies in the decrease of progesterone: as mentioned above, in the dog it appears previous than the occurrence of parturition, whilst in the queen its decrease, although starting before the onset of parturition, is very consistent only after delivery, even in this case with huge individual variations (Johnston et al., 2001a; Siemieniuch et al., 2012). During mid-pregnancy in cats, serum progesterone concentrations were found to range from 34 to 74.4 ng/ml, with a mean value of 46.5 ng/ml in a work from Siemieniuch and coworkers (2012), with huge individual variations. This report was consistent with previous reports (Schmidt et al., 1983). Interestingly, recent findings indicate that placenta is an additional source of P4 in pregnant queens (Siemieniuch et al., 2012). This, besides the importance of characterizing placental

units as a significant endocrine organ, confirm the hypothesis made by Verhage and coworkers in 1976, that observed different concentrations of blood P4 between pregnant and pseudopregnant cats (Verhage et al., 1976). An open question is whether or not this source of P4 is able to sustain pregnancy in case of the removal of the principal, or at least "better known", source of this hormone during pregnancy in cats, i.e. ovaries, after the 45th day of pregnancy (Siemieniuch et al., 2012). Although often disregarded when hormonal assays during pregnancy are concerned, C concentrations in pregnancy fulfill an important role that deserves to be better clarified. In a work from Concannon and coworkers (1978), mean C concentrations in serum of pregnant Beagle bitches was 22.9 ± 1.2 ng/ml around 4 to 2 days pre-partum, with a wide variation ranging between 11 and 43 ng/ml.

During human gestation, increased concentrations of circulating C are reported (Mastorakos and Ilias, 2000). Notably, plasma C concentrations are reported to rise from the beginning of gestation, doubling the titers of non-pregnant women, and then peak during the third trimester reaching three times the previous non-pregnant circulating C concentrations (Mastorakos and Ilias, 2000). An increase during the course of pregnancy was also reported when C was measured in saliva and hair (D'Anna-Hernandez et al., 2011). This maternal rise is part of a more general neuroendocrine change that occurs during pregnancy that aims to allow the fetal development and, at the same time, protect him from detrimental stimuli (Lindsay and Nieman, 2005; Meinlschmidt et al., 2010). Other than that, this elevation has the fundamental function of preparing the mother to the event of parturition (Lindsay and Nieman, 2005; Meinlschmidt et al., 2010). In the last decades, a lot of efforts were addressed to the study of hormonal changes occurring during pregnancy in dogs and, with a lesser extent, in cats. Most of the studies reported in literature, however, have been performed using classical matrices like blood, providing only punctual and short-term information, and with the collection of blood representing a potential stressor for the females. Indeed, the action of sampling could be considered as a stressor itself, thus initiating the production of C that can, in turn, modify the final concentrations detected (Kobelt et al., 2003; Cobb et al., 2016). This was in fact reported for saliva samples collection, in which a little constraint is needed (Kobelt et al., 2003; Cobb et al., 2016). In reproduction, and especially when pregnant subjects and newborns are concerned, every perturbation of the homeostasis could have serious repercussions on the mothers' and newborns' health. Besides this, given the reported length of 60 - 62 days after ovulation for canine pregnancy (Kutzler et al., 2003), the availability of retrospective long-term matrices for the study of this phase could deepen the basic knowledge of this phase and potentially adding new information about it. Other than that, they could provide some reference data

for further comparisons between normal and pathologic pregnancies, representing in turn a feasible tool for the clinician.

Other than pregnancy, the post-partum represents a period full of physical, metabolic, behavioral and hormonal dynamic changes in dogs and cats, so that also this time-window (that lasts about 60 days) deserves further scientific interest. Any possible disruption in the balance of all the factors involved in this complex physiologic phase could have detrimental effects on mothers and offspring. Impairment of reproductive hormones during these two phases, for instance, could lead to pregnancy, parturition, and lactation issues, given their direct and indirect role also on other hormone secretion patterns (Verstegen-Oclin and Verstegen, 2008).

During the post-partum, one of the major physiologic phenomena is lactation, with strong changes occurring at metabolic level, but under control of several hormones. The most relevant metabolic event related to milk production is the energy request. In cats, if during pregnancy the maternal energy demands are 1.5 - 1.7 times higher than the normal basal requests, in the post-partum phase the requests are even 2.5 - 3.0 times higher than in non-reproductive females (Loveridge, 1986). Also in this phase, beside the well-known effects played by prolactin and oxytocin, the high energetic demand is accompanied by an increase in C concentration in queens' blood, that start to rise after parturition, reaching a peak by 4 weeks of lactation (Alekseeva et al., 2020). This hormonal pattern is thought to be functional for the fat mobilization required for the milk production (Alekseeva et al., 2020). In bitches, a work from Cardinali and colleagues (2017) failed in detecting any significant changes of serum C concentration in bitches during pregnancy: they reported a minimum value of 49.8 ± 6.3 nmol/L (about 15.68 ± 1.98 ng/ml) observed at day 30 after ovulation to maximum values at day 45 after ovulation, 72.5 ± 16.1 nmol/L (about 22.83 ± 5.04 ng/ml). On the contrary, a previous study on Beagle bitches reported a peak of C concentrations before giving birth followed by a decrease in its serum titers just after 8 - 12 h post-partum (Concannon et al., 1978). In that study, the mean C serum concentration settled between means of 21.8 ± 1.2 ng/ml and 26.7 ± 5.4 ng/ml throughout the lactation and post-lactation periods, with these constant average concentrations also throughout the weaning phase (Concannon et al., 1978). In both these above mentioned studies, huge individual variations among bitches were reported (Concannon et al., 1978; Cardinali et al., 2017). In humans, C plasma concentrations are reported to increase at parturition and then decrease thereafter, slowly reaching basal pre-pregnant levels (Mastorakos and Ilias, 2000). The same results were reported in women when investigating salivary and hair C concentrations (D'Anna-Hernandez et al., 2011). The biological functions of C during post-partum period, however, could be related not only to the energy

mobilization, but also to the determination of maternal behavior, as reported for hominoid primates (Bahr et al., 1998). In humans, the activation of the HPA axis seems to have an important role in shaping the mother/infant relationship, also because of the effects on the psychological status of the mother (Mastorakos and Ilias, 2000). In a work from Fleming and colleagues (1997) on humans, mothers with higher post-partum concentrations of circulating C were more capable to identify the smell of their own infants. The peak of circulating C seen at parturition in humans, mentioned above, seems to be essential also for the mother-infant bonding (Mastorakos and Ilias, 2000). Other than this, also P4 is reported to be strongly associated with maternal behavior in dogs: its decrease, together with an increase in prolactin, is thought to be fundamental for the manifestation of maternal behavior, including "nesting" (Lezama-García et al., 2019). However, high pre-partum and post-partum stress can contribute to the display of abnormalities in maternal behavior like cannibalism, aggression and rejection of the litter (Lezama-García et al., 2019).

Perinatology in companion animals

In most mammals, the perinatal period is considered as the phase elapsing from the late intrauterine development until the end of the neonatal period (Tønnessen et al., 2012). Its main characterizing event is birth, in which a sudden change from the protected intrauterine environment to the external and harmful habitat is experienced by the newborn (Veronesi, 2013a). In the intrauterine environment, indeed, oxygen, nourishment and metabolite excretion are guaranteed by the placenta, that, in addiction, is a barrier that provides temperature maintenance and mechanical protection from the possible external injuries (Veronesi, 2013a). When the expulsion through the birth canal is completed, the new living-being must provide itself all the vital necessities in a very short time-lapse, through a process named "neonatal adaptation" (Veronesi, 2013a). This process of adaptation, different among the species, is very delicate and crucial not only for the survival in the first hours after birth, but also for the entire neonatal period (Veronesi, 2013a; Morton and Brodsky, 2016). In the process of neonatal adaptation and early neonatal survival after birth, besides neonatal intrinsic factors such as maturity, viability and adequate birthweight, an important role is played by environmental factors, type of delivery and early maternal cares (Lezama-García et al., 2019). This last factor did not receive the needed attention in companion animal reproduction. In these species, indeed, maternal attitude is not fully considered as a

criterion for breeding selection. Therefore, abnormal maternal behavior could represent a not well quoted cause for neonatal losses (Lezama-García et al., 2019).

In companion animals, neonates are born at relatively more pronounced degree of multi-systemic immaturity when compared to, e.g., bovines or equines: it follows that, just after birth, canine and feline newborns are very vulnerable (Veronesi 2013a; Lezama-García et al., 2019). Moreover, canine and feline offspring are classified as "altricial", that means they completely rely on their mother for surviving for a longer time as compared, e.g., to herbivores (Veronesi 2013a; Lezama-García et al., 2019). The process of adaptation in companion animals results therefore slower and less efficient, and every disturbance can create an increased life-threatening risk not only immediately after birth, but also along the entire neonatal period. In this period, newborns are vulnerable to inadequate environmental conditions, neonatal diseases, inadequate nursing and to maternal behavior disturbances.

For these reasons the perinatal mortality rate, including the stillbirths plus losses of newborns within the first 4 weeks of age (Veronesi, 2013a), is very high in companion animals when compared to other species. In fact, although huge improvements in the knowledge about companion animal reproduction, the rates of perinatal mortality are still not tolerable, with values reaching 33-40% in dogs (Tønnessen et al., 2012) and uncertain reports from cats, in which the information are still very scarce. An additional 1.2% of puppy mortality must be considered during the second month of life, when weaning occurs (Lawler, 2008). Therefore, they are very vulnerable not only at birth but throughout the entire neonatal period and even until the end of weaning, that represents itself another transitional, and critical, phase (Peterson, 2011). After the first month of life, indeed, kittens and puppies must cope with another stressor, that is the switch to another type of nutrition, from nursing milk to voluntary eating of solid food (Peterson, 2011). At the same time, the passive immunity acquired by the colostrum intake starts to decrease, increasing the kittens and puppies' vulnerability. Apart of physiology, at the second month of age, puppies can be sold by the breeders to the owners, creating another stressful condition due to the completely new social interactions, environmental and hygienic conditions, that could hamper the health of the puppy and his behavior later in life (Luescher, 2011).

During all these transitions, several mechanisms, prone to guarantee the best survival to the organism, are involved. The most important and recognized until now are related to the activity of the HPA and the HPG axes. About the former, a lot of studies about species different from companion animals were published; about the latter, only few information on dogs and cats are available, despite the recently hypothesized pivotal role during this early phase of development in human medicine (Miller and Auchus, 2011). As mentioned above, the HPA axis is involved in several physiologic processes and its

principal final products are C and DHEA, with C representing the final actor of the mechanism of stress (Compagnone and Mellon, 2000).

This axis is important not only during the last stage of intrauterine development, but also at birth and in the very first moments of extra-uterine life, given that sudden changes in metabolism and a sharply adaptation to the new environment are requested to the newborn. Some findings that C, the final product of the HPA axis, has a role during this mechanism of adaptation are reported in canine newborns. Analyses of C concentrations from coat and claws of dead newborn puppies showed increased concentrations of this hormone in prematurely born newborns when compared to at-term dead newborns or dead puppies in the first 30 days of extra-uterine life (Veronesi et al., 2015). Other than that, another work on fetal fluid concentrations of diverse compounds seems to go in the same direction. Indeed, Bolis and colleagues (2017) found higher amniotic C concentration in the fluids of puppies not surviving at 24 hours after birth. Interestingly, this finding was not detectable in allantoic fluids but, in both types of fluids, an effect of the litter-size on the C concentration was found (Bolis et al., 2017).

In sheep, CRH from placenta stimulates the production of fetal ACTH, that, in turn, leads to an increased secretion of fetal C, that triggers parturition (Mastorakos and Ilias, 2003). This process seems to be initiated by the lack of space that the fetuses experience at the end of pregnancy (Mastorakos and Ilias, 2003). A similar hypothesis was formulated for cats, corroborated by the increase in C concentration in both amniotic and allantoic fetal fluids at term of pregnancy (Fresno et al., 2012). However, no demonstration of this was proven in canine species (Veronesi, 2013b). Other than C, also DHEA exerts important influence on the stress homeostasis (Maninger et al., 2009). Even if not yet fully understood, the role of DHEA-S seems interestingly related to the fetal development; moreover, it is reported to exert anti-glucocorticoid effects (Kalimi et al., 1994). Other than that, short-term administration of prednisone treatment has showed to negatively interfere with the secretion of DHEA, deleting also its circadian physiologic rhythm of production, in young boys (Collomp et al., 2014). This finding once more confirms the interrelation between these two hormones. The anti-glucocorticoid effects mentioned above are mainly orchestrated by inhibition of some types of receptors and enzymes, in turn resulting in antagonize some of the cortisol effects (Kalimi et al., 1994). One of the most important deleterious effects of C is the neurotoxic action that can exert on the central nervous system, especially by its metabolite corticosterone (Maninger et al., 2009). In human species, higher concentration of DHEA are found in brain when compared to plasma circulation, once more confirming its action on the central nervous system (Lacroix et al., 1987). Whilst the increasing

concentration of C in circulating blood before parturition could be addressed to both maternal and/or fetal HPA axis (and in some species also to the placental compartment), DHEA was reported to be highly synthesized by the fetal adrenal gland in humans (Mesiano and Jaffe, 1997). They were additionally reported to be responsive to central ACTH stimulation from the tenth week of gestation (Mesiano and Jaffe, 1997). In a work from Tegethoff and colleagues of 2011, higher concentrations of DHEA, but not of DHEA-S, were found in nails of infants whose mothers were exposed to stressor during pregnancy. The authors suggested that this hormone could possibly represent a marker of stress in perinatal studies (Tegethoff et al., 2011). On the other hand, until recently, fetal gonads were not considered sufficiently mature to synthesize sexual steroid hormones. Interestingly, relying on mRNA expression studies, within the recent years Braun and Jewgenow (2017) reported that gonads of domestic cats are able to synthesize sexual steroid hormones. Other than that, they reported that the gonads have the property of being receptive towards sexual hormones, at least for the period of gestation considered in the study, that went from day 34 to day 48 of pregnancy (Braun and Jewgenow, 2017). This report opens new perspectives for the investigation of the role of the HPG axis during perinatal period.

There is a growing body of research that highlights the importance of the prenatal development in shaping the young and adult periods of life. Starting from the "Barker's hypothesis" (Barker, 2007), this trend in considering the intrauterine life as crucial for the future displaying of various types of diseases has become steady. According to Barker, indeed, low birthweight is associated with poor intrauterine environment and nutrition, and this has a permanent effect in shaping the function and metabolism of a human being, with a specific reference to cardiovascular diseases (Barker, 2007). However, some associations among low birthweight and the influence of sexual steroid hormones in the intrauterine environment exists. Notably, an inverse correlation between circulating testosterone concentrations and birthweight was reported (Manikkam et al., 2004; Carlsen et al., 2006). In sheep, this type of study was conducted administrating exogenous compounds with the aim to mimic the real endogenous T concentrations in subjects that were experiencing normal pregnancies (Manikkam et al., 2004). In humans, instead, some studies were made on women affected by polycystic ovary syndrome, a condition in which the ovaries tend to secrete more T than in physiologic conditions - also during pregnancy state - so that the T concentrations detected were all of endogenous origins (Sir-Petermann et al., 2002; Sir-Petermann et al., 2005). Both in humans and sheep a correlation between higher levels of circulating T and lower birthweight was found (Manikkam et al., 2004; Sir-Petermann et al., 2005). Moreover, other reports that suppose an impact of androgens during intrauterine life on metabolism and

on the future development of cardiovascular disorders exist (Kuijper et al., 2013; Hollier et al., 2014). It is plausible that the sexual steroid hormones concentrations during perinatal life of the fetuses have important implication on the course of life of a living being, including canine and feline species. This furthermore reinforce the necessity of deepening the knowledge about sexual steroid hormones already from prenatal life.

The HPG axis leads to the production of sexual steroid hormones, the most known of which are E2, P4 and T. As mentioned above, only in recent time an interest has grown about the HPG axis and its mechanisms, especially when the role of sexual steroid hormones in the perinatal and early pediatric period is concerned. This renewed interest, occurred both in human and veterinary medicine, brought a new insight about the relationship between the early development of the human fetus and the endocrine system, with a special regard to steroid hormones (Frey et al., 2017). Other than influencing the fetal development and the birthweight, as seen above, and other than their implication in the sexual differentiation process, sexual steroid hormones were reported to affect also the prenatal neurodevelopment (Frey et al., 2017). Frey and colleagues reported, indeed, the importance of studying the prenatal fetal exposure to T for understanding its role in the androgen hypothesis of autism (Frey et al., 2017). In two studies, Hollier and colleagues (2013, 2014), reported a connection between higher levels of cord blood T and increased risk of language delay in human males. The psychiatric sphere is also reported to be directly impacted by the levels of DHEA and T, with a distinct correlation with some disorders (Frey et al., 2017). Language diseases in humans shows a marked difference in terms of prevelance between males and females, and have been ascribed to an overexposure to T during pregnancy (Hollier et al., 2014). In a study of Whitehouse and colleagues (2012), child until 3 years of age were enrolled for studying the development of language delay and data were compared with their hormonal concentrations in cord blood. Results indicated that high concentrations of T during pregnancy are positively associated with language delay in males, whilst on the contrary, it seems that they exert a protective role in females with regard to this disease (Whitehouse et al., 2012). These findings testify the precocious action of HPG axis already from the very first moment of development, and their influence on other fundamental functions of the organisms.

Given the fully recognized importance in the sexually dimorphic health outcomes, the role of the reproductive steroid hormones during the early phases of development, like perinatal and pediatric periods, deserves to be explored in depth, to understand their role and mechanisms of action.

Besides this, deepening the knowledge about these hormones could contribute to assess their physiologic function, thus implying the possibility of identifying new possible endogenous biomarkers

(Frey et al., 2017). In fact, whilst in the past this argument was poorly understood, it is now clear that the developmental plasticity during fetal life deserves further investigations, given its role in establishing future adult health and disease profiles (Barker et al., 2002; Barouki et al., 2012). The influence of the sexual steroid hormones starts in the very early stages of life, i.e. during the intrauterine development (Sathishkumar et al., 2011).

Deepening the knowledge about the influences of the HPG axis on the development already from prenatal life could indeed be useful for at least two reasons: it could help in fully understand the pathogenesis of some important and common diseases and at the same time they could contribute to help in preventing them (Barouki et al., 2012). After the Barker's developmental origins theory of 2007, Barouki and coworkers (2012) reported the necessity of a new approach in prevention of diseases, with a new and wider interest in early development. Literature focused in particular on the difference between the two sexes, comparing the diverse concentrations of sexual steroid hormones in males and females. In human newborns, higher levels of T and of dihydrotestosterone (DHT) were reported in cord blood of males when compared to females (Lundell et al., 2017). On the other hand, Frey and colleagues (2017), reported higher concentrations of DHEA, that is an androgen precursor, in meconium of female newborns when compared to males, whilst T was higher in males than in females (Frey et al., 2017). In humans, an effect of the sex on the gene expression and on the risk of developing certain diseases is reported, but further investigations are needed (Barouki et al., 2012). Other than this, most of the diseases that seem to have a preparation ground in the prenatal life are of major concern from a public health point of view, e.g. type 2 diabetes and hypertension (Barker et al., 2002; Barouki et al., 2012).

Besides this, however, gain further knowledge about this phase could really contribute to understand the perinatal physiology, that is one of the most difficult stage to investigate, not only in human but even more in companion animals, given their very little dimensions. Up to now, indeed, the scarce information available on companion animal newborns have created a difficult access to the knowledge about the physiology of these subjects, even if a prompt approach is fundamental when neonatal patients are concerned (Veronesi et al., 2013a). Furthermore, like Grundy (2006) suggested, it is time to stop think about newborn kittens and puppies as miniature adults and instead try to deepen the knowledge and approach their clinical management on the bases of their peculiar physiologic characteristics. Up to date, very little is known about the role of HPA and especially the HPG axes during this phase, that finely orchestrate this intense and delicate developmental process, representing

an intriguing topic of investigation. In canine and feline perinatology, such information lacks, also because of the difficulty of carry out investigations on those very small-sized subjects.
The real issue is finding matrices that are collectable safely and without invasiveness, limiting the stress and disturbance for the newborns, but also allowing the collection of adequate amounts of samples to allow the laboratory analyses. The perinatal period is therefore still not completely understood, especially in companion animals.

Puberty in companion animals

The beginning of reproduction is marked by the onset of puberty. Puberty is attained through a complex interplay of endocrinological and metabolic factors (Kinder et al., 1995; Hiney et al., 1996; Gobello, 2014). Knowledge about hormonal patterns occurring during the postnatal period were studied in several species, but still today only few data are available about companion animals (Rawlings et al., 2003; Dhakal et al., 2012; Gobello, 2014). Schäfer-Somi and colleagues (2014) stated that "Puberty is defined as the end of a ripening procedure, leading to fully functional primary and secondary sexual organs as well as to typical sexual dimorphic behavior". Puberty occurs thanks to different pathways in the two sexes of canine and feline species. In females, the beginning of puberty is characterized by the occurrence of the first ovulation, while the occurrence of the first spermatozoa in the ejaculate characterizes the starting point of puberty in males (Docke et al., 1981). In pets, puberty is attained on average between 8 and 12 months of age, with a recognized wider interval ranging between 4 and 22 months of age (Peter, 1985; Johnston, 1989). The complex mechanism of the onset of puberty involves several genetic and environmental factors, like body condition, bodyweight, olfactory (pheromones) and visual stimuli (Johnston et al., 2001c).
Puberty attainment is reported to be influenced by bodyweight in feline species: in fact, in both male and female cats, the process of puberty starts when the subject achieves around the 75% of the adult bodyweight (Johnston et al., 2001b). This means that, in most of the cases, the puberty starts when the cat weights around 2.3-4.5 kg (Sojka, 1980; Johnston et al., 2001b). In fact, during this phase, an important influence can be played by the dietary energy availability and, consequently, dietary restrictions could result in delayed puberty onset, as reported for heifers (Perry, 2016).
A fundamental factor for the onset of puberty in cats is the number of daylight hours, that means the reproductive cycle of cats is affected by seasonality (Leyva, 1984; Leyva, 1989; Tsutsui et al., 2004;

England, 2013a). Also in horses, the sexual hormones production and reproductive activity are influenced by seasonality, with higher activity when the season goes towards higher number of daylight hours (Dhakal et al., 2011; Dhakal et al., 2012). Other than that, in both dogs and cats the age at which the puberty starts is reported to be influenced by the breed and, more specifically, by the bloodline (Verstegen, 1998). In cats, this fact is further proved by the different timing in attaining puberty between long-hair breeds (like the Persian cat) and short-hair cats (like Burmese or Siamese cats) (Verstegen, 1998). When comparing different breeds grouping them on the base of the hair length, indeed, Burmese and Siamese cats show a marked precociousness, reaching puberty as early as 3.5-4.5 months of age, with a lighter bodyweight (Verstegen, 1998). This is in contrast with what happens in Persian cats, in which puberty is, most of the time, attained after the first year of life (Griffin, 2002). Another report confirms the influence of the breed, as crossbred cats usually reaches puberty earlier than purebred cats (Griffin, 2002). The genetic heritability of the age at puberty onset has been studied in heifers (Perry, 2016).

In both males and females, however, the pivotal prerequisite for the onset of puberty is the ripening of every element of the HPG axis, gonads included (Alotaibi, 2019). When GnRH neurons have reached maturation, and thanks to multiple physiological conditions and factors, GnRH is released with a pulsatile pattern (Araki et al., 1975; Alotaibi, 2019). After this, through the passage in the hypophyseal portal system, they will lead to the increase in FSH and LH in the anterior hypophysis (Parlow et al., 1964; Desjardins and Hafs, 1968). The pulsatile nature of the secretion of FSH and LH is a consequence of the pulsatile secretion of GnRH (Alotaibi, 2019). After a while, the increase of the amplitude and frequency of pulsatile secretion of GnRH is able to stimulate the hypophysis, that in turn will start to secrete FSH and LH to trigger the gonadal steroidogenesis and gametogenesis, and the increase in the peripheral receptors' uptake (Noakes, 2009; Alotaibi, 2019). It is then clear that even during this important developmental process, an interplay between HPG and HPA axes is seen. In the human species, the maturation of the HPA axis is seen in the first phases of the adolescence, with a phenomenon that is called "adrenarche", an event that leads to an increase in the synthesis of adrenal steroids (Goel et al., 2014). Just after this ripening of the HPA axis, the HPG axis starts to be implicated: an increase in GnRH pulses is seen, and the same cascade reported above is seen also in human boys and girls (Goel et al., 2014). Then, thanks to the increase in circulating estrogens and testosterone, there will be a maturation of the sexual secondary organs, the development of sexual dimorphic behaviors and the closure of the epiphyses (Sjaastad et al., 2013; Alotaibi, 2019). Very similarly, in human, the final rise in sexual steroid hormones is reported to contribute to the maturation

of reproductive tract and its physiology, physical growth, and the appearing of secondary sexual traits (Goel et al., 2014). Other than that, during puberty an age-dependent decrease in the negative feedback of estradiol on LH is observed, leading to an increase in the periodicity of the LH pulses (Sjaastad et al., 2013). From available information in literature it is conceivable that, other than a ripening of the HPA axis, also the HPG axis must complete its ripening process before the onset of puberty (Perry, 2016). The increased secretion of sexual steroid hormones seen during puberty seems to drive other maturational processes, that in turn will shape the responsivity to stress of the HPA axis during the adult life of the subject, so that puberty can be considered as a "key maturational process" for the HPA axis, taking part in its development (Goel et al., 2014).

An interesting report about colts and fillies, sampled by jugular vein, showed that concentrations of sexual steroid hormones were high at birth and then move towards a general decrease until the period around puberty, when their concentrations started to increase again (Dhakal et al., 2012). Notably, around birth, fillies showed P4 concentrations of 5.52 ± 1.77 ng/ml, that dropped the week after to 1.20 ± 0.79 ng/ml and remained at basal concentrations of 0.3 ng/ml from the 3rd to the 47th week of life, when, at the 48th week, showed an increase to 0.97 ± 0.76 ng/ml that reaches 4.10 ± 1.07 ng/ml at the 52nd week (around 13 months of age) (Dhakal et al., 2012). About E2 concentrations, they settled around 153.99 ± 20.12 pg/ml at birth and then reached 58.87 ± 15.10 pg/ml during the 2nd week. After showing the lowest values during middle winter, it started to increase again during spring, in particular during the 47th week of life. During the 50th week, the maximum was reached with a titer of 6.29 ± 33.10 pg/ml (Dhakal et al., 2012). Even in colts, high concentrations of T were reported at birth, with 619.10 ± 152.48 pg/ml that later drop to 90.23 ± 13.28 pg/ml (during the 2nd week). This hormone persisted to be low, around baseline values, until the 47th week, when concentrations of 305.31 ± 38.12 pg/ml were found (during the spring season) (Dhakal et al., 2012). After that, the highest concentrations of T were reached at the 54th week of life, with 578.91 ± 150.55 pg/ml. At birth, E2 showed concentrations of 48.19–291.03 pg/ml in colts, with high variability among the subjects, and then dropped ever after until and during the winter months. After that, a new rise was seen around the time of puberty (Dhakal et al., 2012). Even if performed on horses, this is one of the few works published in literature that focused on the sexual steroid hormones concentrations around puberty, in particular reporting that the concentrations, high at birth, decrease sharply in the fsirst 24 hours of life and then remaining at basal levels until puberty (Dhakal et al., 2012). This trend seems to testify an activation and then a deactivation of the HPG axis, even if the mechanisms underlying this "shutdown" still remain to be clarified (Lanciotti et al., 2018). However, this pattern is also similar to what is

reported in humans, in which the phenomenon of minipuberty is reported (Kuiri-Hänninen et al., 2014; Lanciotti et al., 2018). Indeed, in human babies, postnatal surges of T in males and E2 in females are seen (Lanciotti et al., 2018). Notably, in male babies, T peaks between the 1st and the 3rd month of life, whilst in female ups and downs of E2 are reported until the 6th month of age (Lanciotti et al., 2018). Those peaks result in a gonadal activation in both males and females, and they are considered as a temporary activation of the HPG axis (Kuiri-Hänninen et al., 2014; Lanciotti et al., 2018). This brief activation, however, is parallel to an increase in testis volume and penile growth and to the maturation of ovaria follicles (mostly attributable to the increase in gonadotropins concentrations) (Kuiri-Hänninen et al., 2014).

When puberty is concerned, however, very few data are available on companion animals, especially on dogs. However, a recent work from Calamari and colleagues (2020) investigated the hormonal concentrations of T in blood and hair of Poodle dogs, finding significant differences in the concentration of T in hair of intact males when compared to intact females. Interestingly, no significant differences were found when the comparison involved T concentrations in hair of castrated males and intact females (Calamari et al., 2020). However, in the same work no correlations between the concentrations of T in circulating blood and hair was found, posing the question of the intrinsic difficulty of making comparison between these types of matrices (Calamari et al., 2020). When reproductive hormones concentrations are concerned, however, the results on hair are discordant in brown bears, domestic cats and lynxes (Terwissen et al., 2014; Cattet et al., 2017). In bears, T concentrations in hair of females were higher than in hair of males, however, when data from this species are concerned, seasonality should be taken into account (Cattet et al., 2017). In domestic cats, E2 and T concentrations in hair were found to not differ significantly among the reproductive statuses, even if T concentration in hair was higher in intact than spayed males (Terwissen et al., 2014). Other than this, increasing the knowledge about this phase, and especially a more specific deepening of the interplays occurring during this phase between HPA and HPG axes is strongly recommended (Goel et al., 2014). This necessity seems to be felt, however, in the reproductive medicine of all species. For instance, when heifers are concerned, authors reported the need to better understand the underlying mechanism in the process of puberty attainment, to allow a selection based on the specific genetic mechanisms and involved pathways, with the final aim to improve the reproductive performances (Perry, 2016). In dogs, issues with suspected infertility in dogs that had reached puberty but were sexually immature were reported (Corrada et al., 2006). Indeed, the breeders could face economic losses due to retardation in puberty attainment or even because of the great variability among the

diverse canine and feline breeds in reaching this phase. Thus, more data about hormonal variations and physiology of puberty in companion animals is desirable (Gobello, 2014).

Strengths and weaknesses of different matrices for the investigation of the hormones aim of the study

In literature, hormones are usually investigated using matrices like blood, but, from an ethical point of view, this matrix is not feasible for long-lasting investigations (Russell et al., 2012), especially when the studied subjects are companion animal newborns, too little to allow a suitable blood sampling, or pregnant females, in which any source of disturbance should be avoided.

The first alternatives proposed to blood samplings for research purposes were the feces, urines and saliva because all these specimens allow an easy, repeatable, and without invasiveness collection. However, the main limitation of these matrices is that they report a single-point measurement. When hormones are concerned, blood measures the concentrations related to the exact moment in which the sampling is done. Feces and urine, instead, reflect the concentration of the previous 24-48 or few hours, respectively. They are ideal, therefore, to be used for testing acute changes, but not feasible for the assessment of the overall long-term changes of circulating hormones (D'Anna-Hernandez et al., 2011), which mostly characterize some phases in reproduction such as pregnancy and post-partum, perinatology, and puberty.

When performing hormonal analyses, indeed, the choice of the matrix is crucial, and factors that influence the concentrations of hormones within a matrix must be considered. This was widely studied in humans and, to a lesser extent, in veterinary medicine. In fact, in humans, some hormones like cortisol (C), dehydroepiandrosterone (DHEA) and testosterone (T) are reported to be influenced by many factors (Marceau et al., 2015). One of the most important one is the circadian rhythm. It is known, indeed, that these hormones follow a pattern of daily ups and downs, a mechanism regulated by the suprachiasmatic nucleus and the hypothalamus (Marceau et al., 2015). Salivary C, DHEA-sulphate (DHEA-S) and T are indeed reported to be higher at 8:00 a.m. and then decreasing at 8:00 p.m. and at 2:00 a.m. (Brown et al., 2008), perhaps following a common circadian driver, given that C and T are derivatives of DHEA (Marceau et al., 2015). A role of genetically determined physiological processes in influencing the daily peaks of C was reported (Smyth et al., 1997), even with the possibility of different genes involved in different times of the day (Bartels et al., 2003).

Other than that, it is known that stressors can increase the production of C (Charmandari et al., 2005), both in an acute (Charmandari et al., 2005) and chronic (Baxter-Gilbert et al., 2014; Mack and Fokidis, 2017) manner, creating peaks of C with an acute pattern, or a chronic elevation of the same hormone,

in the second case. It follows that diverse matrices will be suitable to investigate these two different conditions. The choice of the most appropriate matrix is therefore of utmost importance.

For what concerns **blood** specimens, whilst their usefulness for many diagnoses is proved, their feasibility as a matrix for exploring long-term phases is discussed (Mack and Fokidis, 2017). In fact, beyond the reasons explained above, it was reported that the restraint needed for the procedure of blood collection could be addressed as a stressor factor itself, thus increasing cortisol concentrations (Vining et al., 1983; Blackshaw and Blackshaw, 1989; Bennett and Hayssen, 2010). This increase could lead to an overestimation of the C levels (Blackshaw and Blackshaw, 1989; Mack and Fokidis, 2017).

Even if a little restraint is needed also for collecting **saliva** samples, it was reported that up to 4 minutes of restraint do not influence the concentrations of C in the assay (Kobelt et al., 2003; Cobb et al., 2016); however, Oyama and colleagues (2014) reported 2 mins as the time beyond which the concentrations could be modified. Salivary and plasmatic C concentrations are reported to correlate each other, as C levels in saliva reflects the unbound (also defined as "free") fraction that passively diffuses from the acrinar cells of the salivary glands, with the salivary C being 5-10% of the circulating plasmatic C in dogs (Vining et al., 1983; Vincent and Michell, 1992; Cook et al., 1996; Oyama et al., 2014; Cobb et al., 2016). Also in newborn foals, C concentrations were assessed to understand their peak in relation to parturition, and the authors reported saliva as feasible for this kind of study (Nagel et al., 2015). When ACTH was injected in Shetland stallions, a salivary peak of C was detected two hours later, and its return to baseline salivary concentrations was seen only after four hours following the last ACTH administration (Deichsel et al., 2015). As seen in the blood, also salivary C concentrations can fluctuate on a circadian basis, with higher mean concentrations found at 10:00 a.m. when compared to the rest of the day (Beerda et al., 1999). Moreover, also saliva concentrations of C can be affected by stressors, e.g. special restrictions (Breeda et al., 1999), food consumption, and teeth brushing habits (Oyama et al., 2014). Finally, blood contaminations are reported to occur quite commonly. In a work from Wenger-Riggenbach and coworkers (2010), the results tend to show that in a nearly physiologic state, the circulating plasma concentration of C is about 15 times higher than the salivary counterpart. When gross and evident contaminations of blood occurred in saliva samples (that corresponds to approximately an 8% of blood content), the C concentration measured was around 2 times higher than the non-contaminated samples (Wenger-Riggenbach et al., 2010).

Urines were then introduced and tested for their reliability compared to blood serum, especially in humans. A good agreement between the concentrations of 17β-estradiol (E2) in the two matrices, i.e. urine and blood, using gas chromatography with tandem mass spectrometry, was found (Newman et al.,

2019). According to measurement of C concentrations in urines, it was reported that assessing the concentrations of C in the urines every 24 hours could bypass the bias of the circadian rhythm (Burch, 1982). However, an important distinction must be made between urine and blood samples: the latter, indeed, provides the information at the time of the withdrawal, whilst the urines can mirror the concentrations of hormones "accumulated" in a longer previous timespan (Newman et al., 2019); e.g., for progesterone (P4), the concentrations found in the urines were reported to depict the previous 6-8 hours (Newman et al., 2019). Nonetheless, some differences can be found in the results, due to the metabolic processes that occur until hormones excretion in the urines (Newman et al., 2019), other than the possible presence of chronic renal failure or dialysis, reported to impair the use of this matrix in some types of patients (Russell et al., 2012). Even if less invasive, urine collection was reported to be "labor intensive for participants" (Russell et al., 2012), resulting not very feasible for the study of long-lasting periods.

Another matrix taken into consideration in the attempt of studying hormones concentrations without causing stress to the animals, is the **feces**. According to Accorsi and colleagues (2008), indeed, "collection of fecal samples is less stressful than saliva collection, for non-trained animals". For this reason, feces were used to assess the concentrations of hormones in animals, e.g. canids (Sands and Creel, 2004), dogs and cats (Schatz and Palme, 2001; De Palma et al., 2005), wild and zoo animals (Schwarzenberger et al., 1996), sheep (Mostl et al., 2002) horses (Merl et al., 2000) and birds (Touma and Palme, 2005).

In a study on the C metabolites concentrations in feces of dogs and cats, the satisfactory results obtained were accompanied, by contrast, by huge hormonal variations (Schatz and Palme, 2001). In fact, beside the marked differences evidenced between cats and dogs in the production of C metabolites, there were differences in the metabolite excretion between female and male cats (Schatz and Palme, 2001). The enterohepatic circulation, reported to retard the excretion of metabolites in feces (Schwarzenberger et al., 1996), is perhaps one of the reason why C and some metabolites of C were found in feces only 25 hours and 22 hours (medians) after the injection of ACTH in cats and dogs, respectively (Schatz and Palme, 2001). Also in horses, after a painful and stressful situation (castration or colic), higher levels of C metabolites were detected in feces only 24-48 hours after the stimulus (Merl et al., 2000). Differently, another study reported that after ACTH stimulation, higher concentrations of glucocorticosteroids were found 8 hours later in feces of adult caribous (Ashley et al., 2011). Moreover, real variations in the results of hormonal fecal concentrations were reported to be attributable to constipation in some animals (Schatz and Palme, 2001). Even if the concentrations in

feces are not the picture of the instant level of hormones, then, when measures of long periods are concerned, faecal samples may not represent the best option (Accorsi et al., 2008).

Finally, a mention must be done for other matrices collectable without invasiveness, taken into consideration for the study of reproduction and in particular of neonatology.

The first one is the **meconium**, which contains molecules of amniotic fluid (swallowed or inhaled by the fetus during gestation), urines, secretions of the intestines and detached epithelial cells, thus providing a broad spectrum of information about the prenatal life (Frey et al., 2017). Indeed, as its deposition is reported to start around the 13th week of gestation in humans, it is a repository of substances produced by the fetal metabolism, providing information about the fetal development, and could reflect the exposure of the fetus to a wide range of diverse compounds. In a study of Frey and colleagues (2017), the human meconium was pointed out as a useful retrospective matrix, collectable with noninvasive techniques and abundantly available (Ortega García et al., 2006; Frey et al., 2017).

Umbilical cord blood is, on the other hand, another rich-of-information tool for the study of neonatology, reported to give useful information about the concentrations of androgens in the newborn infants (Lundell et al., 2017). Other matrices used for reproduction investigations are the **fetal fluids**, collectable at birth and rich of diverse compounds that could show some information about the prenatal time window (Fresno et al., 2012; Meloni et al., 2014; Bolis et al., 2017; Veronesi et al., 2018). Contrary to older reports on these matrices, they do not only represent the final metabolite synthesis from the maternal metabolism but contains elements derived from the active metabolism of the fetus, as reported for cats (Fresno et al., 2012). Both amniotic and allantoic fluids are, indeed, the reflection of the fetal metabolic activity and the organs development, showing the importance of this matrix for perinatal studies (Fresno et al., 2012). In humans, a deeper knowledge about physiology and composition of fetal fluids was achieved, rendering possible the identification of some markers that are of absolute importance in clinical activity. A similar knowledge and consequent approach are desirable also in feline and canine reproduction, in order to have new basis for the prompt identification of at-risk situations, in this way providing the correct support to newborn puppies and kittens (Veronesi et al., 2018). Amniotic fluid collection via amniocentesis were recently described and proved to be useful for pre-partum verification of fetal lung maturity (Bonte et al., 2017). The authors, however, reported that this procedure has to be carried out very carefully, as it is practicable only on very calm bitches in a dorsally recumbent position, two conditions not always achievable during the last period of pregnancy (Bonte et al., 2017). On the contrary, perform this collection at birth was reported to be a very safe procedure that does not influence the survival rate and welfare of feline and canine newborns and does

not interfere with maternal cares, especially if performed during a caesarean section (Meloni et al., 2014; Dall'Ara et al., 2015; Bolis et al., 2017; Veronesi et al., 2018). For all the above listed reasons, these matrices deserve to be better investigated and their use, especially for perinatal phase of reproduction, should be improved.

Even though those three last matrices are relatively easy to be collected without invasiveness also in companion animals, in all cases the real limitation is the impossible repeatability, thus preventing its use for the long-lasting periods of investigations. Another limitation of all the matrices listed above is their storage. In most of the cases they need to be frozen, requiring specific equipment, thus implying short time and distance between the place of collection and the one of storage. Using those matrices to investigate long-term variations of long-lasting periods as pregnancy, perinatology and puberty, could then result expensive and time-consuming.

Although all these concerns are very important in veterinary medicine, the main consideration regards animal welfare, an ethical issue that has recently gained an increasing importance. The use of noninvasive matrices is aimed not only to prevent biased results, but also to avoid any disturbances to the subjects involved. This is particularly important when phases such as the perinatal period or pregnancy and post-partum are investigated: in these cases, any source of disturbance must be avoid, not only from a "general" animal welfare standpoint, but also because in newborns and in pregnant or lactating females, disturbance can cause excessive or persistent stress that can impair both health and behavior.

In human medicine, with the publication of "The Principles of Humane Experimental Technique" from Russell and Burch, in 1959, a new concept about the involvement of animals in the experimental research was introduced. The "3R" approach was, since then, universally recognized as the framework to approach research involving animals. The 3R rules (Replacement, Reduction e Refinement) refers to the use of animals in research (Russell and Burch, 1959). Even if this principle was originally postulated for the animal use in laboratory researches, the "3R" approach spread also to other different fields of research. The introduction of innovative methods of investigation was reported to be the most efficient strategy for what concerns the "reduction" R (Törnqvist et al., 2014), to which the concept of reducing the number of sampling fit perfectly. The "3R" approach highlights, once more, the importance of the development of new types of investigation, thus leading to explore new matrices for scientific studies also in veterinary medicine.

In the attempt to find a matrix suitable for investigating long-lasting phases, noninvasive in its collection and allowing the reduction of the number of samplings, new matrices were introduced both

in human and veterinary medicine. The aim was to convey the need of avoiding disturbances with the need to obtain the widest range of information from a single sampling. In this sense, matrices that were used with other purposes were recently re-evaluated also for studies about the endocrine patterns of specific phases of life.

For example, **hair** was used for decades to study the exposure to exogenous compounds, and reported to be useful for the detection of doping drugs (Gaillard et al., 1999). Then there was a shift from the detection of exogenous compounds to the endogenous compounds produced by the organism, such as the cortisol in hair, and the mechanism through which the endogenous hormones can be incorporated into the hair shaft was proposed (Gow et al., 2010).

Hair was reported to be used in studies on chronic stress not only in humans (Yamada et al., 2007; Russell et al., 2012), but also in a large variety of animals. More in general, hair proved to be a suitable matrix for the detection of C concentration in wide variety of species, among them: dogs and cats (Accorsi et al., 2008; Bennett and Hayssen, 2010; Bryan et al., 2013; Corradini et al., 2013; Galuppi et al., 2013; Ouschan et al., 2013; Siniscalchi et al., 2013; Svendsen and Søndergaard, 2014; Nicholson and Meredith, 2015; Veronesi et al., 2015; Park et al., 2016; Rosen, 2016; Roth et al., 2016; Willen et al., 2017), bovines (Gonzalez-de-la-Vara et al. 2011; Comin et al. 2012a; Comin et al., 2013; Peric et al. 2013; Moya et al., 2013; Tallo-Parra et al., 2015; Biancucci et al. 2016; Braun et al., 2017; Stradaioli et al., 2017; Fischer-Tenhagen et al., 2018; Tallo-Parra et al., 2018; Endo et al., 2019; Sharma et al., 2019), steers (Baier et al., 2019), horses (Comin et al., 2012b), rabbits (Comin et al., 2012c; Peric et al., 2017), pigs (Bergamin et al., 2019), chimpanzees (Yamanashi et al., 2013; Carlitz et al., 2016), polar bears (Bechshoft et al., 2011), grizzly bears (Macbeth et al., 2010), brown bears (Cattet et al., 2017), black bears (Lafferty et al., 2015), Canada lynxes (Terwissen et al., 2014), chipmunks (Mastromonaco et al., 2014), sheep (Salaberger et al., 2016), australian merino sheep (Sawyer, 2019), wild hyraxes (Koren et al., 2002; Koren et al., 2008), hares (Esposito et al., 2017), rhesus macaques (Davenport et al., 2006), monkeys (Fairbanks, 2011; Feng et al., 2011; Laudenslager, 2011; Dettmer et al., 2012; Meyer and Novak, 2012; Meyer et al., 2014), alaskan caribous and reindeers (Ashley et al., 2011), red deers (Ventrella et al., 2020), coyotes (Schell et al., 2017), siberian flying squirrels (Santangeli, 2019).

The main peculiarity that makes this matrix particularly appealing is the fact that it can provide a long-term endocrine picture of a hormone within an individual (Accorsi et al., 2008).

For what regards the study of the stress, moreover, the effect caused by acute stressors cannot be detected in the hair, as showed in a study in which the injection of ACTH in alaskan caribous was

followed by a peak of glucocorticosteroid concentration in feces but not in hair (Ashley et al., 2011). About the correlation of the concentrations of C in hair and other matrices, some studies reported a positive association between C concentrations determined in hair and in feces (Accorsi et al., 2008), and between hair and saliva (Bennett and Hayssen, 2010), while other authors reported no correlation between hair and feces and between hair and saliva (Bryan et al., 2013).

Most of the studies performed with hair as a matrix were focused on the detection of C, because it is considered a marker of stress, but a preliminary study published by Yang and colleagues (1998) reported the possibility to use this matrix for the detection of sexual steroid hormones, listening some advantages in adopting this choice. Some studies in which sexual hormones were measured in the hair were recently published both in human (Grotzinger et al., 2018; Jahangard et al., 2019) and veterinary medicine (Devi et al., 2018; Tallo-Parra et al., 2018; Bergamin et al., 2019; Ventrella et al., 2020).

Hair sampling is now widely recognized as a noninvasive method, able to overcome some of the major restrictions of the traditional samples when concerning long-term C production (Wosu et al., 2015), but it has displayed to be very risky for canine newborns, as the quantity requested for the analysis with RIA technique was at least 20 mg, thus meaning that the area that needs to be shaved is too big to make this matrix suitable for the analysis in these subjects, given their small size (Veronesi et al., 2015). Another major concern about this type of sampling on newborns is the possibility to damage the fragile skin of the puppies, creating a predisposition for infections and diseases (Veronesi et al., 2015). However, even the hair as matrix of hormones measurement showed some weaknesses. In fact, some factors affecting the concentrations of hormones in the hair matrix were detected and should be carefully considered for a correct understanding of the obtained results (Mack and Fokidis, 2017).

Unfortunately, most of the studies have focused on the factors affecting the concentrations of C in the hair, while very little is known about other hormones.

Hair color

Some studies reported a significant influence of the color on the concentrations of C in hair. Interestingly, most of the studies involving humans did not find significant differences in C concentrations between natural blonde and black hair; on the contrary, the reports about the concentrations of canine coats of different colors differ. Raul and colleagues (2004), Sauvé and colleagues (2007), Kirschbaum and colleagues (2009), and Manenschijn and colleagues (2011) did not

found statistical differences between blonde and black hair in humans. Of these, two studies reported that the results should be interpreted considering that there could have been a statistical underpower of the groups (Raul et al., 2004; Kirschbaum et al., 2009). Another hypothesis underlying this lack of differences is that alkaline substances exhibit higher melanin binding so they are strongly incorporated in brown and black hair, but glucocorticoids are neutral to acidic compounds, so their levels are not expected to be affected by the pigmentation of hair (Dettenborn et al., 2012).

On the other hand, dogs with a black coat were reported to have less C accumulated in hair than their nonblack counterpart, and, within the same subject, black hairs had less concentration of C than agouti and blonde coat (Bennett and Hayssen, 2010). Svendsen and Søndergaard (2014) also found significant differences among three different categories of coat color, but with black color having the highest level of C accumulated, followed by yellow and brown coat. The groups enrolled in this study, however, were not of the same size, and this fact could have affected the statistical analysis, so caution should be taken when interpreting these results (Svendsen and Søndergaard, 2014; Mesarčová et al., 2017). Other authors, on the other hand, found no significant correlations between hair color and concentrations of C in this matrix (Nicolson and Meredith, 2015; Veronesi et al., 2015; Rosen, 2016). Whilst Rosen considered only Border Collies, the study of Nicolson and Meredith considered a pool of dogs of different breeds, so that also in this case the breed factor could have affect the results. Other than this, in grizzly bears, only weak and not significant differences were seen when comparing five different categories of hair color, with black coat having a slightly higher concentration of C than the lighter coats (Macbeth et al., 2010). More specifically, in many bears, the authors reported different concentrations of C when comparing different regions of sampling with different colors and between hair of different colors belonging to the same area of sampling, finding that C tends to be higher in dark than in light hair (Macbeth et al., 2010).

Conflicting results were reported also in the cow. An effect of the hair color on the C concentration in hair is reported, but the extent of this impact seems to change among the different experimental designs (Baier et al., 2019). In some cases, higher concentrations of C were reported in white coat when compared to black coat in cattle (González-de-la-Vara et al., 2011; Burnett et al., 2014; Baier et al., 2019). Conversely, Tallo-Parra and colleagues reported higher concentrations of C in black hair than in white hair (Tallo-Parra et al., 2015). In the same work, however, the authors reported that, whilst the white hair samples were shaved always from the same area, i.e. the frontal region of the head, the black ones were sampled also from the occipital crest. This could represent a confounding factor when interpreting the results, as the place in which the sample is collected could influence the concentration

of hormones detected in hair (Tallo-Parra et al., 2015), as discussed below. In addition to that, even Ghassemi Nejad and colleagues (2017) reported higher level of C in white coats than in the black coats in Holstein cows. More in depth, they observed higher levels of C in hair of cows with a higher percentage of white coat (>85% of coat) than in cows with a mostly black coat (>80%), but did not find any significant difference between black and white hair when compared in general (Ghassemi Nejad et al., 2017). In the same work, serotonin concentrations did not differ between black and white coat both when considering the sample or the entire cow coat (Ghassemi Nejad et al., 2017). A recent work on dogs suggested that, when T concentrations are measured from hair samplings, the color of the coat does not seem to influence the final results in male subjects (Calamari et al., 2020).

The impact of the percentages of various colors in a type of coat should be regarded when performing this type of measurements in mixed colored coat of cattle, for instance when spotted, speckled or roan coats are involved in the research (Baier et al., 2019) and it could be possibly cautious consider the same factor when agouti coat is analyzed in dogs.

Therefore, at present, because of non-consistent results available, the color of the hair should be cautiously considered when the measurement of C is performed in the hair of dogs and cats.

Region of the body

Another factor considered to influence the hormone concentrations in hair is the original localization of the sample collected for the analysis.

Different growth patterns and thus different ways of incorporation of the C levels in the hair shaft could influence the final level of C detected in this matrix (Raul et al., 2004; Dettenborn et al., 2012). The models representing the inclusion of C in the hair shaft suppose a passive diffusion of this hormone from the blood capillaries of the basal membrane to the hair shaft, during the active growing phase of the hair, named “anagen” (Pragst and Balikova, 2006; Comin et al., 2012c). Macbeth and colleagues (2010) found higher concentrations of C in hair collected from the neck compared to the other areas, such as shoulder, rump and abdomen. In reindeers, higher concentrations of C were found in the hair of shoulder and rump when compared to the hair samples taken from the neck, whilst in caribous, shoulder hair had significantly higher concentration of C than the hair taken from neck and rump (Ashley et al., 2011).

A possible explanation could be found in the different patterns of moult, that in turn could influence the concentration of C in the different areas of the coat; other than this, the glandular tissue of some areas could secrete more C, and this could possibly affect the eventual concentration of C in hair of some areas (Macbeth et al., 2010). Furthermore, different rate of growth could be observed in different body sites (Pragst and Balikova, 2006; Svendsen and Søndergaard, 2014). Another consideration reported is that in evaluating C concentration in hair one should considered the different type of hair findable in the different areas of the body, e.g. in bears, with guard hair possibly containing more C than the undercoat (Mesarčová et al., 2017).

However, in eight New Zealand rabbits a work from Comin and colleagues (2012c) did not report differences among different body regions of the same subject comparing 26 different areas of samplings. Another work failed in detecting significant differences between the concentration of C in the hair taken from the two shoulders of the same dog, even if it is important to specify that different timings of sampling were used (i.e. monthly collection for the right side and collection every three months for the left side) (Bryan et al., 2013). In another study on canine subjects, detection of T concentrations in hair was not significantly influenced by the region of the body, when males were concerned (Calamari et al., 2020).

In humans, hair samples taken from the forearms showed higher levels of C when compared with samples taken from the lower legs (Sharpley et al., 2010). In this case the authors speculated that, being the accumulation of C a dynamic process, it could be supposed that different areas of the body have a different response to a stressor, even considering a possible difference between the two sexes (Sharpley et al., 2010). The role of the gender in the different ways of reacting to a stressor, however, was impossible to assess, given the wide range of age between the female and male group, and given the different type of hair sampled. Indeed, the authors reported insufficient amount of hair from the legs and arms of the women, and, on the contrary, a very low amount of sample when hair from the head of men were collected (Sharpley et al., 2010). This latter finding suggests that the area of sampling should be carefully chosen also evaluating the availability of the matrix. For what regards Angus cross beef bulls, indeed, a study indicated that the best zone to harvest hair is the tail (Moya et al., 2013). As a matter of fact, this area showed higher C concentrations in comparison to neck, hip, shoulder and head (Moya et al., 2013). The same finding was reported by Burnett and colleagues: when white hair is concerned, coat from the tail had higher concentrations than shoulders, whereas no significant differences were detected when tail coat was compared with the hip and top line (Burnett et al., 2014).

For its higher concentrations of C, its "easy-to-access" position and the faster pattern of shaft growing, tail is now considered one of the best areas for collecting samples in cows (Burnett et al., 2014).

Washing-out effect

When the results about hormone concentrations in hair have to be interpreted, the washing-out effect was reported as a possible influencing factor, especially when human hair are concerned, because they are usually exposed to a lot of external factors, such as frequent washings, UV rays, cosmetic treatments, that could result in damaging the distal parts of the hair shaft, thus influencing the final concentration of hormones detectable in this matrix, as reported for some drugs (Jurado et al., 1997; Kirschbaum et al., 2009). However, a preliminary study did not find any significant difference in the concentrations of E2, P and T among different segments (top, middle and basal) of human hair shaft (Yang et al., 1998). The same result was found for C concentrations in hair shaft from a group of men and one of women, in which no differences were detected along the hair shaft when considering base, middle and end of the shaft (Sharpley et al., 2010). Other than this, in a work from Davenport and colleagues (2006), no statistically significant differences between the proximal and the distal segments of hair from rhesus macaques were detected. Up to now it is unknown if the hair of dogs and cats could be affected by this phenomenon, but it is possible to speculate that dogs' hair are less subjected to treatments and washings (Corradini et al., 2013). It is than clear that more researches are needed to define the influence of the washing-out effect on the final concentration of hormones, even considering the different laboratory techniques used (Dettenborn et al., 2012).
Moreover, in human medicine hair are not always available, as for example in bald people or in people who refuse to be shaved for religious or cultural reasons (Warnock et al., 2010; Doan et al., 2018).

For all the reasons explained, other alternative matrices were sought. **Nails** were recently reported as a suitable matrix for hormonal investigations, both in human and, as claws and horns, in veterinary medicine (Choi et al., 2001; Warnock et al., 2010; Ben Khelil et al., 2011; Baxter-Gilbert et al., 2014; Comin et al., 2014; Veronesi et al., 2015; Davy et al., 2017; Mack and Fockidis, 2017). This matrix is, indeed, easily collectable, without being a stressor for the dog, and without affecting the external appearance of the subjects (Mack and Fokidis, 2017).

In the last decades, given the dramatic decrease of biodiversity, researches focused on the conservation of species are needed, and the need of long-term programs of preservation is now evident (Hoffmann et al., 2010). The form of threat could be multiple, starting from the human impact arriving to different type of infectious diseases (Baxter-Gilbert et al., 2014; Davy et al., 2017). One of the methods by which it is possible to understand if a population is endangered in its survival is the measure of chronic stress (Davy et al., 2017). Above all the possible detrimental consequences, indeed, chronic stress is related to the impairment of immune and reproductive systems, that are in turn related to the potential of survival of a species (Berger et al., 2005; Baxter-Gilbert et al., 2014). Investigating this, means measuring the retrospective accumulation of stress hormones (Davy et al., 2017). With this regard, claws have been described as a useful method, not only for their peculiarity of long-term retrospective accumulation of hormones, but also for they reported property of being collectable in a noninvasive manner (Baxter-Gilbert et al., 2014).

Nonetheless, claws were reported to be more suitable for the studies on dog puppies, as the amount needed for the sample analysis is lower than the quantity of hair (Veronesi et al., 2015), and their collection do not hamper neonatal health. Notwithstanding the fact that both hair and claws were reported to be useful matrices for the noninvasive investigations on canine long-term hormonal studies, the easier collection of claws compared to hair and the less amount of sample needed make this matrix preferable when enrolling neonatal puppies (Veronesi et al., 2015).

As reported for hair, this matrix incorporates and reflects long-term concentrations of hormones, thus being uninfluenced by stressors that could cause an acute increase and decrease of C (Izawa et al, 2015). For this reason, in human medicine they were used to detect chronic and retrospective concentration of hormones both in adult and neonate patients (Choi et al., 2001; Tegethoff et al., 2011; Doan et al., 2018). In veterinary medicine, some reports of nails used as a matrix for detecting C, and then the status of stress of an animal, exist. Studies have been performed on turtles (Baxter-Gilbert et al., 2014), in bats (Davy et al., 2017), in dogs (Veronesi et al., 2015; Mack and Fokidis, 2017) and in bovines (Comin et al., 2014).

C concentration showed a highly significant positive correlation between coat and claws in a work on canine newborn puppies (Veronesi et al., 2015). Also in a work from Mack and Fokidis (2017) on adult dogs, C concentrations in nails were significantly positively correlated to the C concentrations in hair. The authors, furthermore, detected fewer absolute concentrations of this hormone in nails when compared to hair; they assumed that a possible explanation could be the slower rate of diffusion of C from the capillary vessels into to the nail bed than the diffusion from circulation within the hair follicle

(Mack and Fokidis, 2017). Indeed, the process of hormone incorporation into nail is reported to be a passive diffusion from the capillary vessels into the nail matrix, and then, during the development of the nail, the keratin incorporates the hormone (de Berker et al., 2007).

In veterinary medicine, when nails are concerned, an important distinction among plantigrade animals, like bears, digitigrade, like dogs, and ungulates, like cows, should be made. In fact, the different load of bodyweight on these structures can potentially influence the vascular supply and then result in a different hormone incorporation in different areas of the matrix (Comin et al., 2014). Cows are even-toed ungulates, this means that they use their claws as a support for moving, thus the claws are exposed to an uneven load (Comin et al., 2014). The progesterone concentrations reported by a work from Comin and colleagues (2014) are, indeed, inhomogeneous among the different parts of the sole claw, making this matrix not feasible for the detection of retrospective concentrations of hormones like in other species. In freshwater turtles, Baxter-Gilbert and colleagues (2014) failed in detecting differences between a group of turtles living in a control site or in a road-impacted site. Interestingly, the authors reported significant higher concentrations of C in males than in females. Even if one of the reasons suggested was the lower quantity of females than males enrolled in the study (5 vs. 25, respectively) that could have impaired the statistical evaluation, another possible explanation was the reproductive season of sampling, as it was the post-nesting phase for the females (Baxter-Gilbert et al., 2014). In fact, in green sea turtles a pattern with lower C concentration in post-nesting season, was reported (Hamann et al., 2002). In bats, higher concentrations of C were found in claws from carcasses of bats that have gone through a period of stress due to a fungal infection (Davy et al., 2017). In that case, no differences between the two sexes were reported, and, besides it, the authors did not find any significant difference among the concentrations of claws collected from bats of different geographic regions (Prince Edward Island and Ontario) (Davy et al., 2017). Other than that, in some cases claws were used to assess a medical condition, i.e. hypercortisolism, in dogs (Ouschan et al., 2013).

In literature, discrepancies in the findings between the concentrations of hormones from nails of the right and left hand of humans are reported. Indeed, Higashi et al. (2016) reported significantly different concentrations of DHEA-S between right and left hands of healthy volunteers from both sexes, sampling the nails from the thumb and the forefinger of each hand. They found, however, only slight differences between left and right concentrations of C and T in nails collected with the same method and from the same people (Higashi et al., 2016). Conversely, Voegel and colleagues failed in detecting significant differences between nails clipped from right and left hands of mothers and their newborn

babies when C and P4 were examined, even if a good linear relationship between the concentrations in the two hands was found (Voegel et al., 2018).

Growth rate and type of sampling

About the growth rate, both claws and hair can show differences in the various areas of the body. It is, indeed, reported that different areas of the body could face different rates of hair growth (Burnett et al., 2014; Baier et al., 2019). In fact, in animals, moulting could interfere with the timing of hair growth, as reported for instance in bears, even if the pattern of moulting is reported to be possibly predictable in some species, as the red fox (*Vulpes vulpes L.),* the mink (*Mustela vison*), the European badger (*Meles meles L.*) and the grizzly bear (*Ursus arctos*) (Maurel et al., 1986; Macbeth et al., 2010). Even if, until now, a precise pattern of growth rate has not been defined in beef cattle (Baier et al., 2019), some studies on these animals were carried out. Schwertl and colleagues (2003) reported a monthly growth of tail hair equal to ~ 0.6 - 1 cm in beef cattle, with a daily growth estimated around 0.96 mm per day. Another study, however, reported a growth rate of 0.51 - 0.63 mm/day in Angus and Angus x Charolais cows in the same area (Fisher et al., 1985). The growth of tail hair is, indeed, estimated to be ten times higher than hips and shoulders areas: Burnett and colleagues (2014) reported that, inter alia, hair from the tail switch have an estimated growth rate of 0.51 ± 0.05 mm per day, whilst it was only 0.04 ± 0.05 mm per day for hips and 0.03 ± 0.05 mm per day for shoulders in Holstein cows. The growth rate found in this area, i.e. the tail switch, is the only one that allows intervals of resampling of biological significance in lactating dairy cows (Burnett et al., 2014). Additionally, the growth rate seems to be influenced by some factors. Berman and Volcani reported, in Holstein and Syrian x Holstein cattle, a lower fiber diameter from December to March compared to the rest of the year (Berman and Volcani, 1961). The factor addressed by the authors are not only the day-length but possibly, as hypothesized, the air temperature (Berman and Volcani, 1961).

For what concerns claws, in humans the rate of growth is reported to be around 3 mm/month for fingernails and about 1 mm/month for toe nails (de Berker et al., 2007). To date, to the best of the author's knowledge, no precise information was provided about the growth rate of canine and feline claws. However, interesting anectodical observations indicates the presence of fully developed and formed canine fetal claws around the 30th day of pregnancy (Veronesi et al., 2015). Even if no precise data are available, it is possible to speculate that the growth is influenced by the quantity of movements

made by a subject, the surface on which the movement is performed and the weight of the animal. Other than this, it is possible to suppose a different consumption of the nails, as the forelimbs bear 60% of the bodyweight, whilst the hindlimbs are loaded of the remaining 40% (Carr and Dycus, 2016). About the consumption of the nail, furthermore, it should be underlined that this is another factor that needs be taken into account when hormonal claws concentrations are interpreted. Other than the above-mentioned body weight, also the habits of the subject, the environment and the surface area in which it is accommodated must be considered. It is possible to presume, for instance, that dogs used for work purposes have higher rates of consumption than dogs kept in apartments.

For what concerns cats, it is reported that feline claws tend to grow with a higher rate in the dorsal side than the medial and ventral parts, in this way creating the typical curvature of the claw, with an apico-palmar tendency (Homberger et al., 2009). Even if only anectodically, claws of cats are reported to grow faster than the human ones. Their reported growth rate is 2 mm/week, with a complete turnover occurring in a period of approximately 6-9 months (Erickson, 2013). Another interesting phenomenon that influence the growth rate of the feline claw is the "claw-shedding". First described by De Weerdt, it consists in the loss of the external cornified claw sheath at the same time maintaining intact the internal layers (De Weerdt, 1927; Homberger et al., 2009). This is possible thanks to microcracks that appears only on the external surface without undermine the underlying layers. These little cracks are most likely the result of bending stresses that take place during typical feline activity like prey catching or climbing (Homberger et al., 2009). Sometimes, cats just demonstrate a scratching behavior, for instance on barks, also for territorial purposes, because thanks to this gesture it is facilitated the deposition of the pheromones that are produced from the interdigital glands (DePorter and Elzerman, 2019). Furthermore, it is thought that the claw-shedding is aimed at sharpening the feline claws, that are in turn the principal weapon for the predatory behavior (DePorter and Elzerman, 2019; Homberger et al., 2019). Other than this, they must be able to remove the claws from the prey once captured without the risk of cracking the entire claw sheath, damaging also the vascularized and innervated dermal claw bed and creating a painful and dangerous condition (Homberger et al., 2009). This phenomenon is reported also in anectodical report also in small dogs and horses, but further investigations are needed (Homberger et al., 2009). The hypothesis is that claw-shedding is more diffused than described in the scientific literature, but more diffused in the feline species given their predatory attitude (Homberger et al., 2009). An important difference between dogs and cats, however, lies in the ability to retract their claws. All the felids except cheetah, indeed, are able to retract their claws into protective skin folds when they are not useful for hunting or marking (Homberger et al.,

2009). It is then clear that the mechanisms of consumption between dogs and cats are different and could possibly influence in different ways the final concentrations of hormones findable in this matrix. However, different growth rates imply different times of accumulation, thus creating a "confounding factor" about from how long the hormones are incorporated in a long-term retrospective matrix like coat or claw. In literature, a useful method reported to precisely establish the time of accumulation in hair is the "shave-reshave" method: the area of interest is shaved at the beginning of the observational study, then the next collections will be done according to a precise scheduled time-interval, e.g. 30 days, so that the real time of accumulation is known (Davenport et al., 2006; Meyer and Novak, 2012; Mesarčová et al., 2017; Heimbürge et al., 2019).

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AIMS OF THE PhD THESIS

As above explained, reproduction in companion animals is an intriguing topic of research, that suffers from some lacking knowledge especially in those long-lasting phases. One of the main reasons for this scarcity of information could be addressed to the need of repeating a certain number of samplings requested by longitudinal studies. This need contrasts with the increased interest for animal welfare, in which invasiveness, but also disturbance and restlessness of the subjects enrolled must be avoided. This concern is even more not respected when the repetition of samplings is performed by serial blood collections.

For all these reasons, the availability of matrices collectable without invasiveness and disturbance for the animals, allowing the reduction of the number of samplings, are desirable for all the long-lasting researches involving animals.

Moreover, some categories of animals could be even more sensitive to external disturbances, such as pregnant and post-partum females and newborns.

Thus, in the attempt to comply with the need of respecting animal welfare by reducing the number of samplings and limiting disturbances for the animals for the study of still incompletely explored reproductive phases in companion animals, the project lines of the present PhD thesis were focused to study three most crucial phases of reproduction, that are pregnancy and post-partum, perinatology and puberty by using matrices, such as the coat and the claws, and with a less extent, fetal fluids, collectable without invasiveness.

Each one of the three project lines was therefore scheduled in the above reported three reproductive phases. Notably, for Pregnancy and Post-Partum project line, C and DHEA(S) concentrations were detected in coat of bitches in the pregnant and post-partum phases. For the second project line, Perinatology, C, DHEA(S), E2, T concentrations were detected from claws of newborns from birth until 60 days of age; other than this, another work was aimed to detect the concentrations of leptin in canine amniotic fluid collected at birth. About the last project line, Puberty, E2 and T concentrations were detected from dewclaws and coat of feline subjects from different reproductive statuses and different ranges of age. Other than this, E2, T, C, DHEA(S) were detected from coat of dogs of various reproductive statuses and different ranges of age.

Project line 1

PREGNANCY AND POST-PARTUM

Preface

It was already planned that, when compared to the project line about perinatology, this project line would have required longer time to achieve reliable data from an adequate number of subjects. In fact, although the project started at the first year of the PhD course, the suitable number of dogs from which data were drawn was obtained with some difficulties, due to several factors: notably, two physiologic and one temporal reasons.

The first physiologic factor is the total time elapsing from mating/insemination until the end of post-partum, considered at the end of puppies weaning, at about 60 days of age. Therefore, adding the average 62 days of pregnancy length to the 60 days of puppies weaning, the collection of samples lasted about 120 days for every subject.

The second physiologic factor is related to the possible loss of subjects along the studied period, because not all the inseminated bitches were then diagnosed as pregnant, and not all the diagnosed pregnant bitches eventually whelped.

The temporal factor affecting the current presentation of data about this project line is due to the Covid-19 pandemic emergency that stopped both the on-field and the laboratory activities. It was, in fact, not possible to continue the sampling from the bitches for three months, but also the analyses at the laboratories, in which the access permission was restricted to a limited number of people, reducing the workload. Therefore, some on-field data are not useful (not completed, interrupted) and some of the hormonal analyses scheduled in this project line were not yet carried out, so that final results will be available in the next future, but after the presentation of this PhD thesis.

Moreover, in the cat, after a first compliance of some breeders to enter the study with some females, there were some objections, so that they refused to allow sample collections, thus it was not possible to collect suitable data.

Therefore, this project line is still not completed, and results are showed from the available data obtained in 10 bitches in which cortisol and dehydroepiandrosterone-sulfate were analyzed in the coat.

COAT CORTISOL AND DEHYDROEPIANDROSTERONE-SULFATE CONCENTRATIONS FROM MATING TO 60 DAYS POST-PARTUM IN DOBERMANN PINSCHER BITCHES

Introduction

Success in reproduction is the birth of a viable progeny, at the end of a normal pregnancy. However, differently to horses and cows, in dogs and cats the offspring is defined "altricial", referring to progenies born relatively immature, that continue to be strictly dependent on the mother until the end of weaning (Lezama-Garcia et al., 2019).

Considering the dog, pregnancy lasts about 63±1 day from ovulation, and after parturition maternal lactation is the sole neonatal feeding until about 30 days post-partum, when weaning generally starts, ending at about 60 days post-partum, when puppies are usually sold to the new owners. Therefore, the whole period from mating to the end of weaning lasts about 120 days.

Beside many studies investigated separately pregnancy, parturition and post-partum, the whole period elapsing between mating and the end of weaning did not receive noticeable scientific interest in the dog. This time, in fact, represents a challenging and dynamic period for the female, in which many reproductive, metabolic, emotional and behavioral processes interact (Lezama-Garcia et al., 2019; Santos et al., 2020a).

Differently to cows and pigs, dogs are selected for reproduction upon their genetic and physical characteristics, show/work performances, but very rarely on reproductive/maternal aptitude. It is not uncommon that valuable bitches display troubles in become pregnant and in having a normal pregnancy course, problems at parturition, or issues in providing the normal nursing and maternal cares to the newborns.

Abnormal maternal behavior, leading to insufficient nurse and/or cares to the puppies, aggressiveness against the newborns or cannibalism, can be caused by many factors. Among them, environmental factors, genetics, parity and number of the newborns, were suggested (Lezama-Garcia et al., 2019; Santos et al., 2020a; Santos et al., 2020b). The individual maternal aptitude is therefore the result of many external and intrinsic factors. Among the intrinsic factors, predisposition and positive

experiences are very important, but, as explained above, also the individual extent of the Hypothalamic-Pituitary-Adrenal (HPA) axis activation could play an important role.

Most of the recent studies focused on the role of oxytocin, prolactin and progesterone on the maternal behavior in both humans and dogs (Bridges, 2015; Lévy, 2016; Lezama-Garcia et al., 2019), highlighting the importance of some factors such as parity and litter-size on the hormonal control of maternal behavior (Lezama-Garcia et al., 2019).

The possibility to provide scientific data for a more focused selection of bitches based on reproductive and maternal aptitude could improve the quality of dog breeding (Santos et al, 2020b), maybe concurring to limit the still high perinatal mortality rates, and also in the respect of a more ethical canine husbandry.

Among the constellation of hormones involved in the period surrounding birth, cortisol, estrogens, prolactin and the activation of the oxytocinergic system is pivotal to allow the display of maternal behavior (Lezama-Garcia et al., 2019). Maternal behavior was put in relation to stress and urinary cortisol concentrations in gorillas (Bahr et al., 1998): it was also hypothesized to be associated to maternal stress and was measured through salivary cortisol concentrations in dogs (Bray et al., 2017). These authors found a marginally significant positively association between maternal behavior and higher cortisol levels during the first two weeks after parturition, but did not find association between maternal behavior and the measurement of maternal stress response. Although stress is considered part of the normal preparation to give birth, overstressed females could display the interruption of labor in humans and animals, and, even more, high prepartum and post-partum stress were suggested to be associated to abnormal maternal behavior in some animals (Bahr et al., 1998; Santos et al., 2020a). Lactation and energy expenditure, related to litter-size, can lead to physiologic stress (Alekseeva et al., 2020). Cortisol (C) represents the endpoint of stress, but glucocorticoids are also connected to increased organism load, are good indicators of metabolism intensity, and an increase in the levels of glucocorticoids was also associated to parental efforts (Alekseeva et al., 2020). In female cats, the relationship between C concentrations during lactation and litter-size were studied, and highest blood cortisol concentrations were found 4 weeks after parturition, at the peak of lactation. The authors suggested that measuring C changes could help to understand the reaction of female cats to physiological stress during lactation (Alekseeva et al., 2020). Therefore, an interesting major effect of lactation and kitten nursing on HPA axis activation was evidenced through blood C concentrations.

Dehydroepiandrosterone (DHEA) and its sulfated form (DHEA-S) are precursors of androgens and can be converted in active androgens, and eventually in estrogens (Gabai et al., 2004). However, DHEA is

also recognized to act as a neuroactive steroid with anti-depressant functions, and it is also associated with some behaviors such as sex recognition and aggressiveness (Baulieu, 1998; Zinder and Dar, 1999; Dubrovski, 2000; Maurice et al., 2001). Some studies investigating the DHEA/DHEA-S circulating changes during pregnancy in women (Bird et al., 1980), cow (Gabai et al., 2004), and recently in killer wales (Robeck et al., 2017), reported conflicting results: a decrease of DHEA and DHEA-S in plasma during pregnancy in women; an increase of circulating DHEA-S in early, and even more in mid and late pregnancy compared to pre-fertilization and post-partum in killer wales. In heifers and cows, also, Gabai et al. (2004) reported significant increase of circulating DHEA throughout pregnancy.

About DHEA and DHEA-S analysis, when the technique of analysis is unable to discern between the measurement of DHEA and DHEA-S, the reported results could be referred as the sum of the two compounds and indicated as DHEA(S) (Whitham et al., 2020).

Cortisol and DHEA/DHEA-S could be measured on different matrices, although most studies reported their concentrations and changes measured in plasma. This matrix, however, has two main general limitations: firstly, blood sampling collection could cause pain, fair and restlessness to the animals, not acceptable from an animal welfare standpoint; secondly, when C is assessed, the sampling of blood itself could stimulate the production of C, impairing the evaluation of results. Nonetheless, the measurement of compounds in plasma provides only "punctual" information, so that longitudinal investigation should rely on multiple subsequent samplings, once more conflicting with the goal of respecting animal welfare. On the opposite, other matrices such as the hair (or coat, in animals), collectable without invasiveness, were reported to be useful for long-term, serial, hormonal investigations in a multiplicity of animal species (Heimbürge et al., 2019). Thus, the hair/coat represents a suitable matrix for studying long lasting physiological phases, such as pregnancy and post-partum, in which some long-term hormonal changes occur.

With the aim to provide lacking knowledge about the possible activation of the HPA axis along one of the most challenging and dynamic reproductive phases in female dogs, this present study was focused on the assessment of the possible changes of C and DHEA(S) long-term accumulation in the coat from mating to 60 days post-partum in Dobermann Pinscher, pluriparous, bitches.

Materials and methods

Ethics

The study was performed in accordance with the ethical guidelines provided by the animal welfare committee and all the procedures were carried out according to the Italian legislation about animal care (DL 116, 27/01/1992) and to the European Guidelines on Animal Welfare (Directive 2010/63/EU). A written informed consent was signed by the owners, giving the permission to submit each dog to elective C-section, to collect coat samples, and allowing the record of clinical data for research purposes.

Animals

This study was performed on 10 purebred Doberman Pinscher pluriparous bitches, aged 3-6 years old, belonging to a single breeder. All the bitches showed a history of previous normal pregnancies, post-partum and lactation, and of previous elective caesarean section because of high risk for dystocia due to large breed-related litter-size. The bitches, healthy and submitted to the common prophylaxis, were fed with commercial food, housed in an indoor-outdoor single kennel and handled always by the same operator.

Study design and samples collection

Estrus and mating

From the time of proestrus onset, all the bitches were monitored with vaginal smears, performed every 48-72 hours, and with blood progesterone concentrations, measured every 48 hours from the beginning of the cytologically detected estrus (as evidenced by a vaginal keratinization index above 80%), in order to detect the best time for the sole mating. Natural mating, using a male of proven fertility, was performed only once, 48 hours after the estimated ovulation, as suggested by the detection of plasma progesterone concentrations ranging between 4 and 10 ng/ml (Levy and Fontbonne, 2007). At mating, the BCS of every bitch was assessed on a scale ranging from 1 to 5. The day of the mating, coat was collected by shaving an area of about 5 cm^2 from the dorsal surface of the right forearm. Collection was performed thanks to a gentle handling of the bitch, without real restraint, with a razor (TN2300 Nomad, Rowenta® spa, Milan, Italy), allowing the collection of the coat at the level of the skin. After every coat collection, the razor was disinfected with a 70% alcohol solution. The coat collected was

immediately placed in a paper envelope, closed, and uniquely labeled with an alphanumeric code indicating the subject (alphabetic code) and the time of sampling (numerical code), and stored at room temperature, avoiding any source of moisture contamination. The color of the coat was also recorded and classified as black and rust or brown and rust.

Pregnancy diagnosis and management

At 30 days after ovulation, mated bitches were submitted to pregnancy diagnosis by ultrasound scan and, for the pregnant bitches, the inner chorionic cavity (ICC) was measured as a first calculation of the parturition date (Alonge et al., 2016). Subsequently to pregnancy detection, the second coat sample was collected from the before shaved area, to allow the collection of only the re-grown coat, useful to assess the accumulation of hormones from the previous sampling.

All the pregnant bitches were fully monitored along pregnancy to evaluate the general health, the normal course of pregnancy, and the fetal wellbeing. A second ultrasonographic evaluation was routinely performed at 45 days after ovulation to assess the normal pregnancy course, development and wellbeing of the fetuses, and for the biparietal (BP) measurement, this last parameter useful for a second calculation of the parturition date (Alonge et al., 2016). At this point, no collection of coats was scheduled, to maintain the 30 days-interval design for the collection of samplings.

The sampling time at parturition was not so strictly designed, so that sometimes it was not possible to respect the 30 days of interval after the previous one. In fact, since parturition occurs at about 60-62 days after ovulation, the exact time elapsing between the coat collection at pregnancy diagnosis and the one at parturition could be 30 ± few days length. Collection and storage were the same as reported for the previous sampling time.

Because of the known large litter-size of the considered Dobermann Pinscher bitches, and on the base of higher risk for dystocia when litter-size is > 9 puppies (Cornelius et al., 2019), all the bitches were scheduled for elective caesarean section (ECS) at term of pregnancy. The day of ECS was scheduled on the base of the cumulative evaluation of data (Meloni et al., 2014; Dall'Ara et al., 2015; Bolis et al., 2017; Veronesi et al., 2018): pregnancy length estimated from the date of ovulation, based on plasma progesterone concentrations; days to parturition by measurement of ICC and BP. However, in the last few days before the expected date of parturition, the bitches were daily checked for their clinical conditions and for ultrasonographic fetal wellbeing assessment. Plasma progesterone concentrations were also measured in order to detect the pre-partum blood progesterone decrease, indicative of impending parturition (Concannon, 2000). Only when plasma progesterone concentrations were < 2

ng/ml, ECS was performed. For those bitches in which plasma progesterone concentrations did not decrease to pre-partum levels, recheck was performed until pre-partum progesterone plasma concentrations were detected (Meloni et al., 2014; Veronesi et al., 2018).

Caesarean section and newborn puppies evaluation and care at birth

Elective caesarean section was performed in all bitches with the same anesthetic protocol and surgical procedures, as previously reported (Meloni et al., 2014; Veronesi et al., 2018), aimed to minimize the negative effects on newborns viability.

Immediately after uterine extraction, two expert neonatologists provided assistance to the newborns, within a maximum of 5 min after extraction, evaluating viability of the newborn puppies by Apgar score (Veronesi et al., 2009), and, according to viability classification, submitted to routine neonatal cares or different degree of neonatal assistance, as reported by Veronesi and colleagues (2009). Each newborn was identified with a colored collar and checked for gross physical malformations, gender and weighed before nursing. Mothers and litters were discharged when the mothers were awake and when a normal maternal behavior with puppies was shown, and after having ascertained the presence of mammary secretions.

Data about litter-size, the male-to-female ratio and puppies' birthweight were recorded.

Before surgery, the re-grown coat samples were collected, always from the same area, coded and stored as reported above.

Post-partum evaluation and management

From the day of parturition, a daily telephone follow-up was provided by the breeder. The maternal and litter clinical data and puppies' bodyweight were recorded on a daily basis during the first post-partum week, and on a weekly basis from the second week post-partum until the end of weaning, at 60 days after parturition. Besides it, clinical examinations were scheduled at 1- and 2-week post-partum, and at 30- and 60-days post-partum, for both mothers and puppies. The maternal BCS was re-assessed at 60 days post-partum on a scale from 1 to 5.

Hormone analysis

Coat strands were washed in 3-mL isopropanol to ensure the removal of any steroids on their surface. Coat steroids were extracted with methanol and measured by radioimmunoassay (RIA).

The concentrations of cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEA-S), were measured using a solid-phase microtiter RIA. In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Sciences Inc.) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.5) and incubated overnight at 4°C with 200 μL of the antibody serum diluted 1:20,000 for cortisol, 1:2,000 for DHEA, 1:800 for DHEA-S. The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisol 100%, cortisone 4.3%, corticosterone 2.8%, 11-deoxycorticosterone 0.7%, 17-hydroxyprogesterone 0.6%, dexamethasone 0.1%, progesterone, 17-hydroxypregnenolone, DHEA-S, androsterone sulphate and pregnenolone < 0.01%. The cross-reactivities of the anti-DHEA antibody with other steroids were as follows: DHEA, 100%; pregnenolone, 0.1; dihydrotestosterone, 0.05; DHEA-S, 0.02; testosterone, <0.01%; androsterone, <0.01%; epiandrosterone, <0.01%; estradiol, <0.01%; progesterone, <0.01%; cholesterol, <0.01%; estrone, <0.01%. The cross-reactivities of the anti-DHEA-S antibody with other steroids were as follows: DHEA-S, 100%; androstenedione, 0.2%; DHEA, <0.01%; androsterone, <0.01%; testosterone, <0.01%. After washing the plate with RIA buffer, the standards (5–200 pg/well), the quality-control extract, the test extracts, and the tracer (hydrocortisone {cortisol [1,2,6,7-^{3}H (N)]-}, DHEA [1,2,6,7-^{3}H (N)], DHEA-S [1,2,6,7-^{3}H (N)], were added, and the plate was incubated overnight at 4°C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 μL of scintillation cocktail, the plate was counted on a β-counter (Top-Count; PerkinElmer Life Sciences Inc.).

The intra- and inter-assay coefficients of variation were 3.7 and 10.1%, 3.8 and 10.6%, 3.2 and 11.8, for cortisol, DHEA, DHEA-S, respectively. The sensitivities of the assays were 1.23 pg/well, 0.62 pg/well, 0.54 pg/well for cortisol, DHEA, DHEA-S, respectively.

The sum of DHEA and DHEA-S will be indicated as DHEA(S).

Statistical analysis

An ANCOVA, followed by post Hoc test, was used to assess the possible effects played by sampling time, hair color, parity and litter-size (fixed factors), and by maternal age (covariate) on C and DHEA(S) coat concentrations. Statistical significance was set for $p<0.05$ (JASP®, ver 9 for Windows platform).

Results

From an initial recruitment of 20 mated bitches, 1 was not pregnant at 30 days after ovulation, and in 9 bitches the samplings were stopped at different times because of the Covid-19 emergency rules and lockdown, and were therefore excluded from the study. Therefore, 10 bitches were followed for the entire period elapsing from mating until the end of weaning at 60 days post-partum.

On the collected coat samples, data about C and DHEA(S) concentrations were provided.

All the 10 bitches experienced a normal course of pregnancy, and ECS was performed at 60-63 days after ovulation, providing 10 litters and a total of 103 puppies (1 stillbirth), with litter-size ranging between 10 and 13, and a 58:45 male-to-female ratios.

Data about mean (±SD) maternal age, parity, BCS at mating and at 60 days post-partum, pregnancy length, litter-size, and data about the mean (±SD) puppies' birthweight and Apgar score were reported in table 1.

Table 1 - Data (mean±SD) about maternal age, parity, pregnancy length, BCS at mating and at 60 days post-partum, litter-size, and (mean±SD) puppies' birthweight* and Apgar score* obtained from the 10 bitches enrolled in the present study (* the stillbirth was excluded by the calculation).

Age (ys)	**Parity (n)**	**BCS at mating**	**Pregnancy length (days)**	**BCS at 60 days post-partum**	**Litter-size (n)**	**Birthweight (g)**	**Apgar score**
4.2±1.20	2.3±0.50	3±0	61.2±1.09	2.7±0.24	11.4±1.35	457.7±77.82	8.9±0.66

The amount of coat samples collected was 57.2 ± 29.7 mg (mean± SD), with a range of 18.6 - 86.1 mg.

Coat C and DHEA-S concentrations (mean ± SD) from mating to 60 days post-partum in the 10 bitches enrolled in the study are reported in table 2.

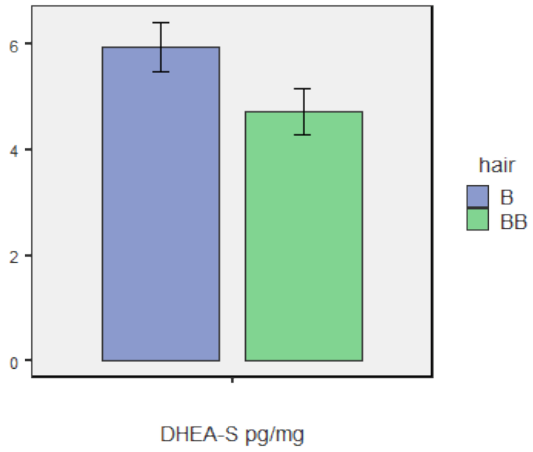
Table 2 - Coat C and DHEA-S concentrations (mean ± SD) from mating to 60 days post-partum in the 10 bitches enrolled in the study

	Mating	**Pregnancy diagnosis**	**Parturition**	**30 days Post-partum**	**60 days Post-partum**
C (pg/mg) mean ± SD	4.98±1.10[a]	5.88±1.83[§]	10.1±3.82	8.94±4.23	10.1±5.14[b#]
DHEA-S (pg/mg) mean ± SD	3.73±1.91	5.01±1.73	6.68±2.23	5.25±2.11	6.12±2.34

[a,b] and [§,#] within rows denotes significant differences ($p<0.01$)

The statistical analysis showed also a significant effect of the coat color on DHEA(S) concentrations, with significant ($p<0.05$) higher DHEA(S) concentrations in black and rust than brown and rust coat (Fig. 1).

Figure 1 – Coat DHEA(S) concentrations in black and rust (blue bar, B) and in brown and rust (green bar, BB) samples



The statistical analysis failed to show significant differences about DHEA(S) concentrations in coat according to sampling time, parity, litter-size. Cortisol concentrations in coat did not differ according to parity, litter-size and color of the hair.

Discussion

This study reported, for the first time, data about the long-term accumulation and changes of C and DHEA(S) concentrations in coat from mating to 60 days post-partum in the bitch, providing some knowledge about the longitudinal measurement of endocrine changes during gestation and post-partum period of the dog, data that are considered to be lacking also in human medicine (Kuijper et al., 2013).
Although previous studies demonstrated the usefulness of canine coat as a matrix for the assessment of both C (Veronesi et al., 2015) and DHEA(S) (Bolis et al., 2015) concentrations, this study provided the first evidence that, if only the re-grown coat is collected, this matrix is suitable for longitudinal studies in which not acute, but chronic, long-term changes are investigated, as previously reported for other species (Comin et al., 2012).
A first consideration should be addressed to the factors related to the coat sampling. The collections of coat were always performed without disturbances or restlessness for the bitches, throughout the procedure, providing a further evidence that this matrix could be collected without invasiveness in the dog. According to the coat sampling schedule, the 30-days apart sampling was designed aiming to a possible balance among the following requirements: a) sampling scheduled at the most important reproductive milestone along the studied phases; b) limitation of the total number of samplings per bitch (therefore reducing the disturbance to the animals); c) sampling times scheduled to assess long-term hormonal changes. However, the designed schedule of sampling time accounted also the need to obtain a suitable amount of coat samples to allow the measurements of hormones. This last need was also ensured by the size of the area shaved initially. An area of about 5 cm^2 was used on the base of previous studies performed on dead newborn puppies (Bolis et al., 2015; Veronesi et al., 2015), in which more than 20 mg of coat sample was obtained shaving an area of about 5 cm^2.
The area of the body in which coat was collected was chosen matching the clinical need for shaving an area of the forearm to allow blood collection for estrus monitoring and for every other clinical indication (blood analyses and surgery). Collecting the coat always from the same body area in all the bitches, however, had also the technical advantage to standardize, as much as possible, the length of the coat collected for the first time and also of the re-grown coat. Besides this, although the bitches belonged to a single breed, in the Dobermann Pinscher two coat colors are recognized by the FCI: black and rust, and brown and rust colors. In the present study, the enrolled bitches were numerically equally distributed in the black and rust (5 bitches), and brown and rust (5 bitches) varieties of coat color. The statistical analysis surprisingly showed significantly higher DHEA(S) coat concentrations in

black and rust than in brown and rust samples. The colors of the coat were reported to influence the accumulation of hormones like cortisol (Bennett and Hayssen, 2010; Svendsen and Søndergaard, 2014), while other studied did not find differences related to the color of the coat (Nicolson and Meredith, 2015; Veronesi et al., 2015; Rosen, 2016). The result of the present study highlights, once more, that the role of the color on hormonal concentrations in the coat needs further and more focused investigations.

About the selection of bitches, some precautions were adopted. Firstly, only one canine breed was selected, to limit the possible differences of maternal behavior related to the breed (Santos et al., 2020b), genetic-based influence on HPA activation and on circulating hormones concentrations (Potischman et al., 2005), and on the growth rate of the coat. However, it is well known that, even within a breed, genetic lines or inter-individual differences can be observed, especially when HPA axis activation is concerned.

Secondly, to avoid the influence of environmental/managerial/nutritional confounding effects, all the bitches belonged to a single breeder and were handled by the same operator. On the other hand, it could be interesting to investigate the role of diverse canine breeds and managerial environmental conditions on the long-term HPA axis activation during pregnancy and post-partum.

From a reproductive standpoint, all the enrolled bitches were pluriparous, so that the possible impact of the first experience of mating, pregnancy, parturition (although by ECS) and puppies grooming and nursing was avoided (Lezama-Garcia et al., 2019). Also in this respect, further investigations deepening the role of the condition of primiparous on C and DHEA(S) accumulation in the coat could better clarify the effect of a new maternal experience on the extent of HPA axis activation, as demonstrated for maternal behavior (Santos et al., 2020b). Moreover, all of the enrolled bitches showed a normal pregnancy course and were in good general health conditions at term, and also at the end of weaning 60 days post-partum (as evidenced by the BCS at the end of weaning). Even the prerequisite for performing the ECS, and all the practical factors related to ECS (parturition date calculation, pre-partum monitoring, anesthetic protocol, surgical team, post-surgery management), were the same in all the bitches, avoiding possible influence of several factors on the HPA axis activation. Therefore, although the factors that could be involved (and interact) in the process(es) of the HPA axis activation and, consequently, on the C and DHEA(S) production and their accumulation in the coat, are many, the major possible precautions to avoid "on-field" influences were adopted.

When data obtained about the retrospective and long-term accumulation of C and DHEA(S) in coat are concerned, some interesting considerations arose.

Because of its retrospective significance, the concentrations measured at mating should be considered as the starting point for the evaluation of possible hormonal changes. Therefore, the concentrations detected in the coat samples collected at pregnancy diagnosis indicate that, when long-term accumulation of hormones in the coat are considered, no significant C and DHEA-S changes seem to occur within the first 30 days of pregnancy. A significant increase of C was detected between mating-pregnancy diagnosis and 60 days post-partum, suggesting that the HPA axis is "gradually" activated, and that the higher concentrations of C in coat detected at 60 days post-partum could be the result of the complex interaction of events that simultaneously occur, such as uterine involution, grooming and nursing of the puppies, and lactation; this, indeed, implies physiological, metabolic, emotional, and behavioral changes for the bitch. Unfortunately, it is impossible to discern if (and which) one of those physiologic processes could be the major cause of the observed accumulation of C in coat at 60 days post-partum.

Lactation, puppies grooming and nursing are continuous and dynamic complex processes, in which stressors could continue to stimulate the HPA axis, leading to a chronic C secretion that, in turn, leads to a continuous accumulation of hormones in the coat. This is particularly interesting, especially when considering that only pluriparous and experienced bitches, with normal maternal behavior, were enrolled. This result, therefore, could be considered as a starting point for future investigations on the possible effect played by maternal parity (and many other maternal factors) on the changes of C concentrations in coat during the post-partum period. Nonetheless, this result could also represent an important base for the future assessment of maternal attitude and for a better selection of bitches for reproduction. The observation of significantly higher C concentrations in coat at 60 days after parturition, on the other hand, represents a limit of the present study. Due to the retrospective significance of the data, it should have been interesting, in fact, to prolong the study to, at least, 90 and 120 days post-partum, to detect if, and when, the concentrations of C in coat would have returned to the initial values. This could be interesting not only to complete the hormonal profile study, but also to assess the possible effect of puppies and of weaning (physical removing by the mother because of selling to the new owners, causing the end of nursing and possible emotional changes) and variation of metabolism (due to the end of lactation), on HPA axis activation with the consequent secretion and accumulation of C in the coat.

The absence of significant changes of DHEA(S) concentrations in coat during the studied period is surprising. Although the investigation of DHEA/DHEA-S plasma levels during pregnancy provided conflicting results in women, cow and killer wales, DHEA was reported to act as neuroactive steroid

involved in behavioral circumstances (Baulieu, 1998; Zinder and Dar, 1999; Dubrovski, 2000; Maurice et al., 2001), implicated in the stress response (Balieu, 1998; Zinder and Dar, 1999; Dubrovski, 2000), and reported to stimulate marked lobuloalveolar mammary gland development in rats (Sourla et al., 1998).

Another consideration regards the lack of effect played by maternal parity and litter-size on the long-term accumulation of hormones in coat. The enrollment of bitches belonging to a single large breed, characterized by large litter-size, positively reduced the variability of results, but on the other hand, prevent the assessment of the possible effect of diverse litter-sizes on HPA axis activation, that deserves scientific interest. In a study from Alekseeva and coworkers on cats (2020), the maternal experience factor did not play a significant role on C concentrations in blood, but, on the other hand, circulating C tended to be higher in females with small litters (with up to 3 kittens) at 4 weeks of age. Furthermore, gestational profiles of C concentrations were not affected by age or parity in the bottlenose dolphin (Steinman et al., 2016), and in the killer whales (Robeck et al., 2017).

Moreover, in the present study, all the puppies were normal and viable at birth, with a birthweight within the standards and showed a normal growth in the 60 days after birth according to the breed (unpublished data), so that the results obtained refer to mothers with normal litters. It could be interesting, therefore, to investigate the effect of less viable or weak/diseased litters on maternal HPA activation.

Conclusions

In conclusion, the results from the present study report, to the author's knowledge, the first data about changes of long-term retrospective concentrations of C and DHEA(S) in coat of pluriparous bitches belonging to Dobermann Pinscher breed. The finding of higher C concentrations in coat at 60 days post-partum than those assessed in the coat collected at mating and at pregnancy diagnosis, seems to suggest that the complex interaction among physiologic, emotional and behavioral events in the post-partum period plays a central role in the HPA axis activation and C secretion and accumulation in coat. DHEA(S) did not show significant differences related to time sampling, but black and rust coat showed higher DHEA(S) concentration than brown and rust samples, suggesting new and more focused investigations about the effect of coat color on C and DHEA(S) accumulation in canine coat. Neither maternal parity, nor litter-size influenced C or DHEA(S) coat concentrations.

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Project line 2

PERINATOLOGY

Preface

In this project line three studies have been performed and results published on international peer-reviewed, high IF and Q1 journals.

In the first two studies, cortisol (C) and dehydroepiandrosterone-sulfate (DHEA-S), and 17β-estradiol (E2) and testosterone (T) were respectively measured on claws serially collected from birth to 60 days of age in puppies. Both studies were aimed to investigate the role of the main hormones of the Hypothalamic-Pituitary-Adrenal (HPA) and Hypothalamic-Pituitary-Gonadal (HPG) axes during the perinatal period.

The use of claws was suggested by a previous study from Veronesi and colleagues (2015) that proved the suitability of this matrix for studying retrospective long-term hormones accumulation and its preference when compared to the hair, the collection of this last resulting as dangerous for the possible damages to the newborn fragile skin. Moreover, the very small amount of sample proved to be suitable for hormone analysis, as previously reported by Veronesi and colleagues (2015).

In dogs, the first appearance of claws is anecdotally reported to be around 30-35 days of pregnancy (Veronesi et al., 2015). The samples collected at birth, therefore, represents the hormones accumulation occurred in the second part of the fetal intrauterine life. The samples collected at 30 days of age monitor the changes occurring during the first month of age, in which the newborns face a lot of transitional and challenging processes. The samples collected at 60 days of age reflect the weaning and social phase of interaction of puppies.

The studies reported in the present PhD thesis once more demonstrated the usefulness of claws for the longitudinal study of retrospective, long-term accumulation of hormones in puppies. On the base of the "clip-reclip" rule, re-grown claws showed to be collectable without invasiveness and damages, every 30 days, allowing the measurement of hormones accumulated within a scheduled time-window.

In the first study a significant trend of C and DHEA(S) decrease was found from the sample collected at birth (thus providing data about the hormones accumulated from the intrauterine first appearance of claws in the fetus until the time of collection at birth) as compared to the following samplings at 30 and 60 days after birth, and also between 30 and 60 days of age. Moreover, an effect of the type of birth (vaginal delivery vs elective caesarean section) was played on both hormones on the samples collected at birth, with higher concentrations observed in puppies born by vaginal delivery than caesarean section, suggesting a different HPA activation in the puppies based on the type of birth.

In the second study, a significant decrease of E2 and T was seen from birth to 30 and 60 days of age, but not from 30 to 60 days of age. Greater T concentrations were found in males when compared to females, with an interaction between sex and sampling time. The Apgar score was positively related to T concentrations in claws collected at birth, while the bodyweight was positively correlated with T concentrations, with an interaction among puppy sex, bodyweight and sampling time. Results indicated that there are greater E2 and T concentrations at birth compared with 30 and 60 days of age, and this could be the result of the influence of prenatal sexual steroids during fetal development.

The third study was carried out using a different type of matrix, the collection of which is, also in this case, performable without invasiveness, but providing only a single time measurement: fetal fluids.

This kind of matrix was evidenced as a useful tool for the study of perinatology also in dogs, as previously reported in humans and other animals. A study from Meloni and collegues (2014) reported the higher IGF-I concentrations in amniotic than allantoic fluid, and the lower IGF-I amniotic concentrations in small and medium size bitches when compared with large ones, while no differences were found in allantoic IGF-I concentrations among size groups. NEFA did not differ between the two fetal fluids, but higher NEFA concentrations were found in small as compared to medium and large breeds. The results, therefore, indicated a relation between IGF-I and NEFA concentrations in fetal fluids and breed body-size.

Therefore, a further study focused to deepen the possible effect played by breed size on amniotic leptin concentrations in dogs was performed during the PhD course, and results are presented below.

Because of the recognized role of leptin as a key factor not only on the energetic homeostasis, but also at multiple levels, influencing the control of reproduction, food assumption and metabolism, the study aimed to assess the amniotic fluid leptin (AFL) concentrations at term of pregnancy in healthy dogs. The results showed that AFL concentration was significantly higher in small-sized puppies in comparison to large-sized puppies, suggesting an influence of breed body-size on fetal metabolism, as previously reported for NEFA and IGF-I.

The usefulness of claws collected without invasiveness for cortisol and dehydroepiandrosterone (sulfate) monitoring in healthy newborn puppies after birth

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Abstract

Despite the high rates of perinatal mortality in dogs, the study of canine perinatology did not receive strong scientific interests until recently, probably due to the difficulties in studying long term changes evaluations without incur with ethical issues. In the recent years, however, the usefulness of new matrices, such as the claws, collectable without invasiveness and providing information about a long-term, retrospective window of time were reported to be a suitable tool for the study of canine perinatology in spontaneously dead puppies. The present study was therefore aimed to assess the usefulness of claws as matrices collectable without invasiveness for the study of immunoreactive cortisol and dehydroepiandrosterone (sulfate) in alive puppies at birth, at 30 and at 60 days of age. Beside the effect played by the sampling time, the possible effect of type of birth, vaginal delivery (VD) or elective Cesarean section (CS), as well as of some other maternal or neonatal factors was assessed. The results showed a significant decrease of both hormones from birth to 30 and to 60 days of age ($p<0.001$) and from 30 to 60 days of age ($p<0.05$), and highlighted, for both hormones, the influence of the type of birth, with newborns born by VD having higher concentrations of immunoreactive cortisol ($p<0.01$) and deydroepiandrosterone (sulfate) ($p<0.001$) than the newborns born by elective CS in collections made at birth. No other significant effect was detected. The study confirmed the usefulness of claws as matrix collectable without invasiveness for the retrospective, long-term assessment of hormonal changes in alive newborn puppies and that both hormones declined from birth to 60 days of age. The differences between puppies born by vaginal delivery or elective Caesarean section suggest a possible different HPA activation in puppies born by the two types of birth.

The usefulness of claws collected without invasiveness for cortisol and dehydroepiandrosterone (sulfate) monitoring in healthy newborn puppies after birth

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1. Introduction

The perinatal period is the phase that lasts from the final intrauterine fetal stage of development until the end of the neonatal period. Unfortunately, until recently, the study of canine perinatology did not receive large consideration, despite the high perinatal mortality rate, reported to reach values as high as 20-40% [1,2]. The perinatal period is a very complex, transitional phase, in which the fetus must prepare itself for birth and for the neonatal life in the extrauterine environment. Therefore, a variety of structural and physiological changes occur from the last intrauterine stage of development until the end of the neonatal period. However, there is a growing body of evidence that the perinatal period could have also a role in defining the healthy outcomes during the adulthood [3,4]. Most of the changes that occur during this phase are controlled or associated to hormonal variations. Among all the hormones involved in this transitional phase, cortisol (C) and dehydroepiandrosterone (DHEA), secreted by the Hypothalamic-Pituitary-Adrenal (HPA) axis, play a central role. In particular, C has been reported as one of the most important hormones during the perinatal period, given its role during the last stage of final intrauterine fetal development and maturation of several organs [5], to trigger the process of parturition in humans [6,7], as well as in ruminants [8] and to drive the transition from the intrauterine to the extrauterine life [9]. DHEA is known to exert anti-glucocorticoid effects, principally mediated by enzyme and receptors inhibition, providing a balancing role against a wide range of cortisol effects, the most known of which is the neurotoxic effect of corticosterone, a cortisol metabolite; also DHEA has a recognized neuroprotective role [10,11]. Before birth, circulating C levels can result from the hormone production by the fetal

and/or maternal HPA axis and, in some species, also by the placenta, whilst DHEA was reported to represent the major steroid produced by the fetus itself [12]. Once produced, DHEA is quickly metabolized to the sulfated form, DHEA-S, reported to be more stable [11]. Therefore, the study of both hormones can provide more accurate information about the prenatal steroid fetal HPA activity. Cortisol and DHEA were traditionally studied on plasma/serum, but alternative biological matrices, collectable without invasiveness, were recommended to avoid the confounding effect of venipuncturing and to respect animal welfare. As alternatives to blood sampling, therefore, the use of feces, urines and saliva were reported to be more suitable for C analysis in humans and several animal species. The concern about blood sampling is particularly recognized by human and veterinary neonatologists, and become even more important in small animal neonatology, due to the very small size of newborn dogs and to their fragile veins. However, the use of alternative matrices such as feces, urines and saliva, providing punctual information, requires frequently repeated samplings, and this does not accomplish the need of limiting the possible disturbances to the newborns. Other than that, measurements of hormones concentrations in saliva, urine and/or feces can suffer by some disturbing factors, such as the type and the time of food assumption and the circadian rhythm [13-15], as reported in humans and dogs. For those reasons, the long-term studies on perinatology should rely upon the use of matrices that allow the reduction of the number of samplings [15] and provide a stable accumulation of hormones. In the recent years, many studies [16,17] reported the usefulness of the hair for retrospective studies about C changes in humans and animals, thanks to the characteristic long-term, stable accumulation of this hormone in hair. The concentration of C in coat samples was measured also in canine spontaneously dead newborns [18], providing first data about the different C coat concentrations in dependence of age at death and demonstrating the utility of the coat also for studies about canine perinatology. However, that study highlighted also the main disadvantage about the use of the coat in newborn dogs, represented by the actual amount of sample necessary to allow the analysis, about 20 mg. To reach this quantity, the coat has to be shaved from a large area, unacceptable in living newborn puppies because of the consistent risk of creating injuries to their fragile skin and of impairing newborn's health by predisposing the puppy to infections. Additionally, in veterinary medicine some works reported an influence of the hair pigmentation on the C concentration detected in this matrix [19], while others reported no differences [18,20]. Finally, the hair follicle was reported to be a possible local source of C production; therefore, the total C measured in this matrix may originate either locally or systemically [21-23].

For these reasons, according to previous studies performed in humans, using fingernails as alternative to the hair for the retrospective analysis of C and DHEA/DHEA-S concentrations [24-27], the usefulness of claws in dead puppies was

59 tested [18]. Claws were found to be easily collectable and characterized by a consistent reduction of the total amount of
60 sample to allow the C analysis in comparison to the coat (about 5 vs 20 mg, respectively). Therefore, the authors
61 concluded that claws represented a suitable alternative to the coat for studies on living puppies. However, one limitation
62 of that study was the lack of DHEA/DHEA-S analysis, that prevented to understand what is the actual production of the
63 hormone by the fetuses.
64 In canine husbandry, very often canine breeders use to cut the tip of the puppies' claws at birth to avoid scratching of
65 the maternal mammary glands skin that can lead to discomfort during suckling or even lesions with
66 inflammation/infections. Thus, tips of claws can be easily and without invasiveness repeatedly collected from newborn
67 puppies along the time between birth and selling (after 60 days of age) and used for research purposes.
68 Therefore, given the importance of researches about canine perinatology performed using matrices collectable without
69 invasiveness and with the lowest disturbances for the newborns, the present study was aimed to assess C and DHEA-S
70 concentration in the claws collected from alive puppies from birth to 60 days of age, evaluating possible changes related
71 to age and also the possible effect played by some factors, such as the maternal age and parity, the litter-size,
72 birthweight, Apgar score, newborn gender, type of parturition, times for birth, order of birth.
73
74 **2. Materials & Methods**
75
76 *2.1 Animals and clinical data*
77 The study enrolled 13 large-size (body weight ≥ 20 kg) purebred, healthy bitches, belonging to some breeds (Maremma
78 Dog, n=3; German Shepherd, n=2; Béarnaise Mountain Dog, n=3, Leonberger, n=1; Saint Bernard, n=2; Rottweiler,
79 n=2), monitored from the time of the sole mating, based on blood progesterone analysis, until parturition and puppies
80 weaning. Parturition dates were calculated on the basis of plasma progesterone concentrations at mating, coupled to the
81 ultrasonographic measurement of the inner chorionic cavity at about 25-28 days after mating [28] and again by the
82 ultrasonographic measurement of the fetal bi-parietal diameter at 40-45 days after mating [28]. For 8 pluriparous
83 bitches, based on the previous history of troubles at parturition, elective Caesarean section (CS) was planned, while the
84 other 5 bitches (3 primiparous and 2 pluriparous) were monitored and surveilled for spontaneous vaginal delivery (VD).
85 In the last days of pregnancy, all the bitches were daily monitored for appearance of clinical signs of impending
86 parturition, while the candidates for elective CS were also submitted to plasma progesterone analysis and to the
87 ultrasonographic evaluation of fetal heart rate and well-being. Elective CS was therefore performed also on the basis of

clinical records and when progesterone plasma concentrations were <2 ng/ml. The chosen anesthesia protocol was mainly aimed to avoid negative impacts on the newborns [29]. Briefly, after premedication with atropine (0.02 mg/kg IM, Atropina Solfato®, Fatro Spa, Ozzano dell'Emilia, Italy) and metoclopramide (0.2 mg/kg SC, Vomend®, Eurovet Animal Health B.V., Bladel, The Netherlands), antibiotics were administered (cefazolin 25 mg/kg IV, Cefazolina TEVA®, Teva Italia Srl, Milan, Italy) before the beginning of surgery, and anesthesia induction was obtained with propofol (4-6 mg/kg IV, Proposure®, Merial Italia Spa, Milan, Italy), followed by maintenance with isofluorane (Isoflurane Vet®, Merial Italia Spa, Milan, Italy) in oxygen. The Cesarean section was performed with a ventral midline laparotomy, followed by lidocaine splash (lidocaine 2%, 2 mg/kg, Lidocaina 2%®, Esteve Spa, Milan, Italy) on surgical incision at the end of the surgery. As soon as the fetuses were removed, tramadol (3 mg/kg IV, Altadol®, Formevet Srl, Milan, Italy) and oxytocin (0.15 UI/kg IM, Neurofisin®, Fatro Spa, Ozzano dell'Emilia, Italy) were injected to the mothers. The times elapsing between anesthetic induction and the first and the last fetus extraction were recorded. For those bitches whelping by spontaneous vaginal delivery, the time from the beginning of the second stage of parturition (considered as the time from the first observed strong abdominal contraction) to the first fetus expulsion, the time elapsing between each fetus expulsion, and the time from the beginning of the second stage of parturition and the last puppy expulsion, were recorded. From now on, all these intervals will be generally cited as "times for birth". In all the cases, once born, the puppies were evaluated by Apgar score system [30] and a clinical examination was performed to ascertain the absence of gross physical defects or malformations. Birthweight and gender of the newborns, as well as the litter-sizes were also recorded, and for those puppies born by VD also the order of birth was recorded. Only normal, viable and normal weighed (according to the breed reference ranges of the Italian Kennel Club-Ente Nazionale Cinofilia Italiana) puppies were enrolled in the study and individually identified by differently colored strings used as collars. Maternal data, such as breed, age, and parity were also recorded. Bitches were clinically monitored until puppies weaning for normal postpartum progression, and puppies monitored during the 60 days after birth for normal development and weight gain.

In addition to the informed consent to allow anesthesia and surgery for those bitches undergoing CS, all the breeders signed an informed consent to allow the collection of tips of claws from their puppies and the use of data for research purposes.

2.2 Claws collection

In order to standardize the method of claws collection, in all the cases claws were collected always by the same author

(JF). Within 12 hours after birth, the tip of each claw was clipped and the single puppy pooled claws sample was stored in individual paper envelope, at room temperature, until analysis. At 30 and 60 days of age, only the re-growth claws were clipped and collected; this part was recognizable because lighter and smaller than the remaining body of the claw. Every time once collected, the single puppy pooled claws sample was labeled and stored as reported above.

2.3 Cortisol analysis

Claws were washed in 5 mL isopropanol to reduce at minimum the risk of extracting C from the outer part of the nails and to assure the removal of any steroids or other contaminants from their surface. The minced samples were extracted in a glass vial with methanol and were incubated at 37° C for 18 hours. Then, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved of phosphate buffered saline (PBS), 0.05 M, pH 7.5. All the samples were frozen dried as reported by Comin et al. [31], and their dried weights were calculated.

The claws immunoreactive cortisol concentrations were determined using a solid-phase microtiter RIA, as described by [18,31]. The rabbit anticortisol antibody used was obtained from Biogenesis (Poole, UK). Cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8%, and aldosterone <0.02%. Intra- and inter-assay coefficients of variations were 3.7% and 10.1%, respectively. Sensitivity of the assay was 1.23 pg/well and it was calculated as the interpolated dose of the reaction to a concentration of zero minus the statistical error.

DHEA(S) analysis

Regarding the analysis of DHEA(S), because the antibody showed a noticeable cross-reactivity for both DHEA and DHEA-S, the hormonal concentrations reported in this study have to be regarded as "immunoreactive DHEA(S)".

The claws immunoreactive DHEA(S) concentrations were measured using a solid-phase microtiter RIA. In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Sciences Inc., Milan, Italy) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.4) and incubated overnight at 4°C with 200 µL of the antibody serum diluted 1:80,000. The cross-reactivities of the antibody with other steroids were as follows: DHEA-S, 100%; DHEA, 100%; DHEA 3-glucuronide, 15%; androstenedione, 5.9%; pregnenolone, 0.3%; epiandrosterone 3-glucuronide, 2.7%; androsterone sulfate, 2.9%; cortisone, < 0.001%; cholesterol, <0.00001; cholesterol oleate, <0.00001. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts, and tracer (DHEAS

146 [1,2,6,7-3H (N)]; Perkin-Elmer Life Sciences; specific activity: 70.5 Ci/mmol; 20 pg/well) were added, and the plate
147 was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells
148 in RIA buffer. After addition of 200 µL of scintillation cocktail, the plate was counted on a β-counter (Top-Count,
149 Perkin-Elmer Life Sciences, Milan, Italy). Intra- and interassay CV were 3.2 and 11.8%, respectively. The sensitivity of
150 the assay, calculated as the interpolated dose of the response to a concentration of zero minus the statistical error, was
151 0.54 pg/well. To determine the parallelism between standards curve and endogenous immunoreactive DHEA(S), in
152 claws containing high concentrations of endogenous hormones, samples were serially diluted in 0.05 M PBS, pH 7.5.
153 The relationship between claws immunoreactive DHEA(S) concentrations and the standard curve determined through
154 linear regression was linear: the correlation coefficient (r) was 0.99 and the model was given by the equation
155 y=0.99x+0.28.
156
157 *2.4 Statistical Analysis*
158 Data were firstly analyzed for normal distribution by Shapiro-Wilk test and then statistically analyzed by ANCOVA test
159 aimed to evaluate the effects played by fixed factors, such as the sampling time (birth, 30 and 60 days of age), the type
160 of birth (elective CS or VD) and newborn gender (male or female), and by covariates, such as maternal age and parity,
161 litter-size, birth weight, Apgar score, and "times for birth" according to VD or elective CS on immunoreactive C and
162 DHEAS claws concentrations. The post hoc test was used to better investigate the effect of each sampling time on claws
163 hormonal concentrations. For the puppies born by vaginal delivery also the order of birth was considered as a covariate.
164 The possible correlation between immunoreactive C and DHEAS claws concentrations at each sampling time was also
165 assessed by Spearman correlation test. Significance was set for $p<0.05$ (JASP, ver 9 for Windows platform).
166
167 **3. Results**
168
169 *3.1 Clinical findings*
170 The 13 bitches gave birth to 75 puppies, 73 of which were normal developed, normal weighed and viable, while 2 were
171 born dead. The maternal and neonatal clinical data, recorded at birth, about the 73 healthy puppies grouped according to
172 the type of birth (vaginal delivery or elective Caesarean section), are reported in table 1.
173
174 Table 1. Maternal and neonatal clinical findings about the 73 healthy puppies, grouped according to the type of birth.

175 Data are expressed as mean ± SD.

	Vaginal delivery (n=28)	Elective Caesarean section (n=45)
Maternal age (years)	3.8 ± 1.7	4.1 ± 2.4
Maternal parity	2.1 ± 1.6	2.7 ± 1.5
Litter size (n)	7.1 ± 1.0	6.4 ± 2.9
Birthweight (g)	570.5 ± 112.5	552.1 ± 120.1
Apgar score	9.0 ± 0.7	8.1 ± 0.4
Gender (M/F)	12/16	25/20

176

177 In the bitches submitted to elective Caesarean section, the mean ± SD times elapsing between anesthetic induction and
178 the first and the last fetus extraction were 11.7 ± 1.2 min and 19.6 ± 7.5 min, respectively. For the bitches whelping by
179 spontaneous vaginal delivery, the mean (± SD) time from the beginning of the second stage of parturition to the first
180 fetus expulsion was 80 ± 32.4 min, the mean time elapsing between each fetus expulsion was 168 ± 45.6 min, and the
181 time between the beginning of the second stage of parturition and the last fetus expulsion was 308 ± 110.0 min.

182

183 *3.2 Cortisol and DHEA(S) claws concentrations*

184 Because all the bitches showed a normal post-partum and all the 73 healthy puppies showed a normal weight gain and
185 development during the following 60 days of observation, claws samples were available and analyzed from all the 73
186 puppies at birth and at 30 and 60 days of age.
187 The statistical analysis showed a significant effect of the sampling time (birth, 30 and 60 days of age) on both
188 immunoreactive C and DHEA(S) claws concentrations, and the post hoc test evidenced that for both hormones the
189 claws concentrations at birth were higher than those recorded at 30 and 60 days of age (C: 26.8 ± 16.2 vs 9.8 ± 4.5 and
190 5.5 ± 3.2 pg/mg, respectively; DHEA(S): 211.7 ± 146.9 vs 96.1 ± 72.4 and 61.9 ± 48.3 pg/mg, respectively) ($p<0.001$),
191 and that the concentrations recorded at 30 days were higher than the ones recorded at 60 days of age (C: 9.8 ± 4.5 vs 5.5
192 ± 3.2 pg/mg, respectively; DHEA(S): 96.1 ± 72.4 vs 61.9 ± 48.3 pg/mg, respectively) ($p<0.05$) (table 2).

193

194 Table 2. Immunoreactive Cortisol and Dehydroepiandrosterone (sulfate) concentrations (pg/mg) in claws collected at 0,

195 30 and 60 days of age from the 73 puppies enrolled in this study.

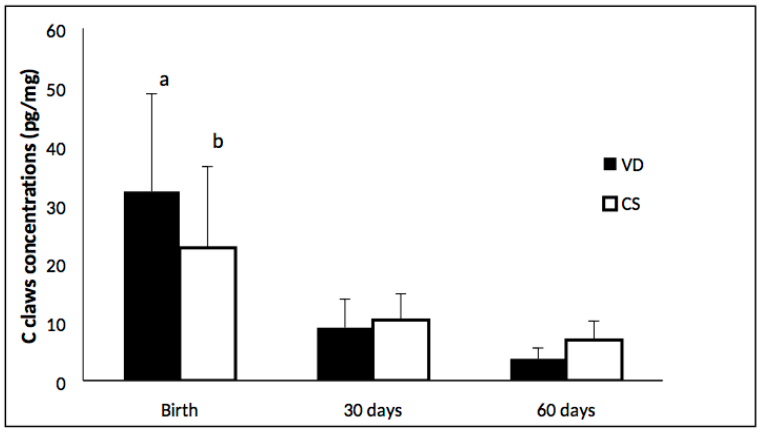
	Birth	30 days	60 days
Cortisol (pg/mg)	26.8 ± 16.2[a]	9.8 ± 4.5[b]	5.5 ± 3.2[c]
Dehydroepiandrosterone (sulfate) (pg/mg)	211.7 ± 146.9[a]	96.1 ± 72.4[b]	61.9 ± 48.3[c]

196

197 [a,b]p< 0.001, [b,c]p< 0.05 within rows
198

199 However, the ANCOVA test showed also the significant effect played by the type of birth on both immunoreactive C
200 (p<0.01) and DHEA(S) (p< 0.001) claws concentrations only at birth, with higher concentrations in claws of puppies
201 born by VD than elective CS (VD vs CS: C mean ± SD, 32.2 ± 16.6 vs 22.6 ± 13.8 pg/mg, respectively; VD vs CS:
202 DHEA(S) mean ± SD, 300.5 ± 146.2 vs 164.9 ± 111.3 pg/mg, respectively). No significant effects of the newborn
203 gender or of the other covariates (maternal age and parity, litter-size, Apgar score, birthweight and times for birth) were
204 found. The Spearman correlation test showed that immunoreactive C and DHEA(S) claws concentrations were
205 significantly correlated at birth (R= 0.45; p<0.001), and with a borderline significance at 60 days of age (R= 0.26;
206 p=0.05), but not at 30 days of age.
207 Data about the immunoreactive C and DHEA(S) claws concentrations, expressed as mean + SD in puppies born by VD
208 and elective CS are showed in Figs 1 and 2, respectively.
209

210 Figure 1 - Immunoreactive cortisol (C) concentrations in claws (mean + SD) in puppies born by vaginal delivery (VD)
211 or by elective Cesarean section (CS) collected at birth, 30 and 60 days of age.

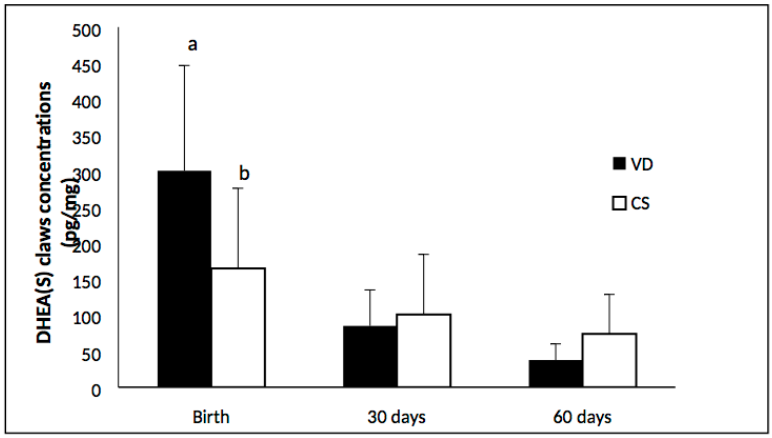


212

213 [a,b]p< 0.01

214 Figure 2 - Immunoreactive dehydroepiandrosterone sulfate concentrations in claws (mean + SD) in puppies born by

215 vaginal delivery (VD) or by elective Cesarean section (CS) collected at birth, 30 and 60 days of age.



216

217 [a,b]p< 0.001

218

219 **4. Discussion**

220

221 To the best of the author's knowledge, apart from some preliminary results [32,33], this is the first study in which the

222 results about the serial collection of tips of claws from alive puppies for the study of immunoreactive C and DHEA(S)

concentrations around birth are reported. In the present study, the collection of the tips of claws showed itself to be easy, non-invasive and to require only a very limited restraint. Furthermore, this procedure is considered positive by the breeders to accustom the newborn puppies to the first human handlings. Moreover, the minimum amount of sample requested for the analysis, i.e. 5 mg, furthermore demonstrated the usefulness of the claws matrix also in alive very small sized animals, as previously suggested in dead puppies [18].

Another important issue in favor of the claws matrix is related to the long-term accumulation of hormones, previously reported in some studies [18,25] that limits the number and frequency of collections and prevent disturbance of mothers and puppies. In the present study only three collections were performed with intervals of 30 days. This time span was chosen for three reasons: to limit the number of collections, to allow a realistic time for claws re-growth, and to respect a reasonable constant interval for hormones measurement. About the last criterion, indeed, it was anecdotally reported [18] that fully formed fetal claws are usually visible from around the 30^{th} day of pregnancy. Therefore, the hormones analyzed in claws collected at birth depict their accumulation occurred between the last 30 days of pregnancy and birth, while the hormones analyzed at 30 days on the claws re-growth report the accumulations occurred between birth and 30 days of age, and the hormones analyzed at 60 days on the further claws re-growth report the accumulations occurred between 30 and 60 days of age.

A limitation of the current study could be represented by the limited number of puppies included. However, because C was reported to be higher in small breed dogs [34], only one breed body-size was considered and the choice of select only large-size breeds dogs was related to the possible technical advantage of greater quantity of tips of claws collectable in comparison to small-sized newborn dogs.

The present study showed that both the mean immunoreactive C and DHEA(S) concentrations in claws significantly declines from birth to 30 and again to 60 days of age, depicting a trend of decrease, with a significant positive correlation between the two hormones at birth. The same trend of claws C concentrations decrease was previously reported also in dead puppies [18], even if the mean C concentrations at birth reported from those authors were higher in comparison to those found in the present study (62.6 ± 59.0 pg/mg vs 26.8 ± 16.2 pg/mg, respectively). Because variations of C are associated with the probability to survive of the newborns [35], this difference may be explained by the fact that the present study enrolled only viable and healthy puppies. However, in both studies it is possible to observe a progressive decrease of C claws concentrations. This trend of C claws concentrations decrease seems therefore to suggest that the activity of the fetal HPA-axis is higher in the last month of canine pregnancy in comparison to the subsequent neonatal period, even if the immunoreacive C concentrations in claws at term may not necessarily be

caused only by higher production in the fetus. In fact, because of the claws' permeability, their "incubation" for a long period in the amniotic fluid could have also influenced the accumulation of cortisol metabolites. However, as previously stated [18], analyzing C concentrations in the claws of puppies does not allow to discern the source of C production, and the final hormone accumulated in the fetal claws could be the result of maternal, fetal or maternal-and-fetal production. For this reason, in the present study, parallel to claws immunoreactive C concentrations, also immunoreactive DHEA(S) claws concentrations were analyzed, because it was recognized that DHEA, and therefore DHEA-S, better represents the HPA activity of the fetus itself [26]. The finding of also higher immunoreactive DHEA(S) claws concentrations at birth seems to support the hypothesis that, at least in part, the canine fetus itself is responsible for the higher C and DHEA-S production that accumulated in the fetal claws during the second half of pregnancy, highlighting, once more, the important role of these hormones for the final stage of fetal development and preparation for birth. In fingernails collected from human neonates 0 to 21 days old, DHEA-S mean concentration was 123 pg/mg [26]. Although this value is lower than the one found at birth in the present study, a direct comparison between the two studies is not possible. On one hand, indeed, the different results could be addressed to the different species studied, on the other hand the time of collection in the human neonates was spread in a long interval so that the result could not be compared with the findings in newborn dogs at birth or 30 days of age because our samplings were not made during a time span but at established, fixed times.

The higher immunoreactive C and DHEA(S) claws concentrations found at 30 than at 60 days of age seems to suggest that during the first 30 days of age the HPA-axis of the newborn puppy is less stimulated in comparison to the last stage of intrauterine life, but still higher activated in comparison to the time elapsing during the second month of age. This finding seems to be reasonable, because during the first month of age, the newborn dog must cope with the complex process of neonatal adaptation and multi-organ final maturation, while during the second month of age, most of the organs and apparatuses are largely developed and functioning.

Other parameters, such as maternal age and parity, litter size, neonatal gender, type of birth, Apgar score, birthweight, the times for birth according to the type of parturition were reported or supposed to possibly influence C and/or DHEA-S concentrations in biological specimens, and therefore also in the present study, beside the sampling time, these parameters were studied in order to verify a possible influence of them on immunoreactive C and DHEA(S) claws concentrations in puppies. Moreover, although all the enrolled puppies were normal developed, weighed and viable, also the birth weight and the Apgar score were statistically assessed, and for the puppies born by spontaneous vaginal delivery, also the order of birth was considered. Apart from the type of birth, none of the studied parameters had a

281 significant influence on immunoreactive C and DHEA(S) claws concentrations along the time of study. Surprisingly,
282 only on samples collected at birth, both immunoreactive C and DHEA(S) claws concentrations were higher in puppies
283 born by spontaneous vaginal delivery in comparison to those born by elective Caesarean section. This finding seems
284 therefore to suggest that, also when elective CS is planned to be performed as closest as possible to the physiologic end
285 of pregnancy, but before the bitch enter in labor, the fetal HPA axis is not as highly activated as in those fetuses
286 undergoing the final preparation for birth by vaginal delivery. This result is even more interesting in consideration of
287 the long-term hormones accumulation measured on claws collected at birth, suggesting that the great activation of the
288 fetal HPA-axis in the dog fetuses seems to occur just in the last hours before birth. The absence of immunoreactive C
289 and DHEA(S) claws concentrations differences according to the order of birth in puppies born from spontaneous
290 vaginal delivery is also interesting. It could be supposed that these hormones measured in claws collected at 30 days of
291 age could have been different in first or last born in comparison to litter-mates. The lack of significant differences could
292 be however explained as the absence of different HPA activation according to order of birth in parturition processes
293 occurred within short time, as demonstrated by the intervals recorded between the beginning of the second stage of
294 parturition and the first fetus expulsion, the interval among subsequent fetuses expulsion and between the beginning of
295 parturition and the last fetus expulsion, always within the reference ranges [36]. Another possible explanation could be
296 addressed to the small number of total litters enrolled in the present study and especially those born by spontaneous
297 vaginal delivery and, therefore, this topic deserves further investigations on a larger number of litters.
298 About the times for birth, different parameters were considered according to the type of parturition. However, in the
299 cases submitted to elective CS the times for birth were always very short and in agreement with data reported in
300 literature [37,38]. Also for those puppies born by spontaneous vaginal delivery, the times for birth were always within
301 the ranges reported by Linde-Forsberg and Eneroth [36].
302 Among the other parameters, the absence of significant influence played by maternal parity on immunoreactive C levels
303 is in agreement with what is reported for rhesus monkeys' hair [39]. On the opposite, the absence of significant
304 differences in both immunoreactive C and DHEA(S) claws concentrations according to newborn gender, disagrees with
305 the results reported by Kapoor et al. [39], that found higher DHEA concentrations in the hair of females than in males
306 of neonate monkeys, but it is in agreement with data reported on C claws concentrations in spontaneously dead
307 newborn puppies [18].
308 As a first study, the present results were drawn only from healthy puppies, providing first, preliminary results mirroring
309 the normal conditions, adding new lacking information about canine perinatology. From a practical point of view,

however, it will be very interesting to collect data about other body-size breeds and especially from low viable/diseased puppies.

5. Conclusions

In conclusion, the present study provided a first evidence of the usefulness of newborn puppies' tips of claws as a valuable, non-invasive collectable matrix for serial studies about the perinatal hormonal changes. The results showed that immunoreactive C and DHEA(S) display a positively correlated trend of decrease from birth to 60 days of age and that the type of birth is characterized by different immunoreactive C and DHEA(S) claws concentrations at birth, suggesting a different HPA activation in puppies born by elective Caesarean section in comparison to those born by spontaneous vaginal delivery.

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Perinatal concentrations of 17β-estradiol and testosterone in the toe claws of female and male dogs from birth until 60 days of age

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Perinatal concentrations of 17β-estradiol and testosterone in the toe claws of female and male dogs from birth until 60 days of age

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ABSTRACT

This study was conducted to assess concentrations of 17β-estradiol (E2) and testosterone (T) in toe claws of puppies collected at birth, at 30 and at 60 days of age, evaluating changes relating to age and effect of puppy sex, Apgar score, bodyweight at birth, "litter effect", litter size, and maternal age. Puppies (n = 89), 46 males and 43 females, with normal weight and without malformations, were assigned for the study. Within 12 hours of birth tips of toe claws were clipped, and the re-growth tissue of the claws was collected at 30 and 60 days of age. Steroid quantifications occurred using a radioimmunoassay. The results indicated there were lesser concentrations (P<0.001) of both hormones at 30 and 60 days of age than at birth and that concentrations were similar at 30 to 60 days of age. There were greater (P<0.001) T concentrations in males than females, with there being an interaction between sex and sampling time (P<0.01). The Apgar score was positively (P<0.001) related to T concentrations in toe claws at birth. The bodyweight was positively correlated (P<0.05) with T concentrations, with an interaction among puppy sex, bodyweight and sampling time (P<0.05). Results of the present study confirmed the usefulness of toe claws as a matrix for study of hormonal changes in perinatology of dogs. Results of the study also indicate there are greater E2 and T concentrations at birth compared with 30 and 60 days of age that could be the result of these prenatal steroids affecting fetal development.

Keywords: Dog; Perinatology; Sex steroids

1. Introduction

The time elapsing between the last stage of fetal development and the end of the neonatal period is known as the perinatal period. This period is very complex and characterized by dynamic, long-lasting changes, difficult to study using traditional matrices, such as blood, because of invasiveness of repeated samplings. This is particularly important in dogs, in which the important variables during the perinatal period have seldom been evaluated. In recent years, however, interest

in the study of the perinatal period of dogs has increased, because of both a desire to develop a greater understanding of the biology during this developmental phase in dogs, and also the potential importance for late health outcomes reported in the humans (Kuijper et al., 2013). In addition to the perinatal functions of the hypothalamic-pituitary-adrenal (HPA)-axis, and of cortisol, the effect of the hypothalamic-pituitary-gonadal (HPG)-axis and sex steroid hormones are also recognized as being important. This is especially the case with the early development of the fetus and regulation of sexual differentiation, and the effects on the neural development of the subject (Kuijper et al., 2013; Hollier et al., 2014; Frey et al., 2017). In addition, adult health profiles also seem to be affected by the early effect of hormones, in particular behavior, cardiovascular and psychiatric disorders, and metabolism (Kuijper et al., 2013; O'Connor and Barrett, 2014). Many diseases are reported to be sexually dimorphic in late adulthood, but the effects of sexual steroids are initiated during the perinatal period.

The functions of steroid hormones in the perinatal and early pediatric periods (Frey et al., 2017) in humans and animals, has been recently recognized. Most of the available data were drawn from studies performed in humans and focused on the function of androgens, and especially of testosterone, because of its early production by the testes during fetal development. Estrogens, however, have also been recognized as having functions in developmental programming, although there is less known in this regard as compared with the androgens.

Because, as reported in humans (O'Connor and Barrett, 2014), after birth the circulating concentrations of sex steroids are usually less, than those present at birth, until puberty. The sex differences observed subsequent to birth, therefore, may be attributed to prenatal sex steroid effects. Results of studies with horses indicate concentrations of circulating sex steroids are relatively greater before birth, decrease immediately after birth, and increase again at puberty (Dhakal et al., 2012).

The authors are not aware of any studies investigating the perinatal concentrations of sex steroids in newborn dogs, most likely because of the difficulties and invasiveness related to blood sampling of neonatal puppies. Invasiveness represents a limitation in all research on humans and animals, especially when neonatology and perinatology are concerned. Another limit of those matrices is that they provide only short-term information, and the impossibility of repeating collections. Recently, matrices such as the hair/coat and the nail/claw tissues, have proved suitable for retrospective, long-term investigations into hormone accumulation - especially for the study of perinatology in newborn babies and dogs (Tegethoff et al., 2011; Veronesi et al., 2015; Fusi et al., 2018). At the time of birth of puppies, claw collection proved to be safer and more useful than the collection of hair, avoiding any injury to the fragile skin of the newborn puppy (Veronesi et al., 2015; Fusi et al., 2018).

Considering the importance of studying perinatology using matrices that can be safely collected, the present study was conducted to assess the concentrations of the primary sex steroids: 17β-estradiol (E2) and testosterone (T) in the toe claws of puppies collected at birth, at 30 and at 60 days of age. Possible changes in hormone concentrations as related to age were assessed along with the effects of other factors such as the puppies sex, Apgar score, bodyweight recorded at birth, 30 and 60 days of age, "litter effect", litter size, and maternal age. The "litter effect" is considered to be a possible factor resulting in variation among litters (i.e., factor effecting differences between puppies from different bitches).

2. Materials and methods

2.1. Animals and clinical data

The study was approved by the Università degli Studi di Milano ethical committee (OPBA) with protocol OPBA_147_2019.

There were nine German Shepherd bitches (4-6 years old) assigned for this study, belonging to a single breeder, that were healthy, and pluriparous (two to three previous parturitions). Bitches were monitored from before the time of the single mating until the time of weaning the puppies, at 60 days after whelping. In all cases an elective Cesarean section (CS) was planned, for the safety of bitches and puppies. The CS were performed on the basis of the medical history (dystocia at the previous parturition) and based on causes not related to the present study that focused on quantitation of hormone concentrations such as fetal malposition, fetal anasarca, and extended parturition duration with fetal death. The date for surgery was planned and timed based on factors that have been previously reported (Meloni et al., 2014; Fusi et al., 2018).

The estimated date of parturition was calculated on the base of the sole mating, scheduled when the quantities of plasma progesterone concentrations was 8 ng/ml or greater, quantifications occurring using ELFA (enzyme-linked fluorescence assay) in a MiniVidas automated analyzer (Biomerieux®, France; Brugger et al., 2011). The second factor that was considered in determining the time of the CS was the measurement of the inner chorionic cavity (Luvoni and Grioni, 2000) performed 25 to 28 days after mating and by determining the bi-parietal diameter (Luvoni and Grioni, 2000) at 40 to 45 days after mating. The third criteria used to determine the time of the CS were findings during the last week before the expected date of whelping when all of the bitches were monitored daily to identify possible clinical signs of approaching parturition. The elective CS was performed on the basis of the estimated date of parturition based on the date of mating combined with the clinical maternal and fetal monitoring, and determinations as to when progesterone plasma concentrations were < 2 ng/ml. To minimize all possible negative effects on the neonatal puppies,

there was a specific anesthesia protocol used (Fusi et al., 2018). Premedication was performed using atropine to limit oral secretions (0.02 mg/kg IM, Atropina Solfato®, Fatro Spa, Ozzano dell'Emilia, Italy) and metoclopramide (0.2 mg/kg SC, Vomend®, Eurovet Animal Health B.V., Bladel, The Netherlands), antibiotic prophylaxis was performed using cefazolin (25 mg/kg IV, Cefazolina TEVA®, Teva Italia Srl, Milan, Italy), and the induction of anesthesia occurred using propofol (4-6mg/kg IV, Proposure®, Merial Italia Spa, Milan, Italy) and was maintained with isofluorane (Isoflurane Vet®, Merial Italia Spa, Milan, Italy) in oxygen. The CS procedure was initiated with a ventral midline laparotomy and after completion of the CS there was a lidocaine splash applied (lidocaine 2%, 2 mg/kg, Lidocaina 2%®, Esteve Spa, Milan, Italy) on the site of the initial incision. Immediately after the extraction of the last fetus, tramadol (3 mg/kg IV into the cephalic vein, using Altadol®, Formevet Srl, Milan, Italy) and oxytocin (0.15 UI/kg IM into the semimembranosus/semitendinosus group of hindlimb muscles, Neurofisin®, Fatro Spa, Ozzano dell'Emilia, Italy) were injected into the bitches. Each puppy was evaluated for viability using the Apgar score system (puppies' heart rate, muscle tone, and other signs to ascertain if extra medical care or emergency care is needed; Veronesi et al., 2009), and underwent a clinical examination to ascertain the absence of anatomical defects or gross physical malformations, by visual evaluation. Data such as puppy sex, birthweight, and litter size were also recorded.

Only normally developed, healthy, viable (Apgar score $\geqslant 7$) puppies of normal weight (according to the reference range for the breed of the Italian Kennel Club-Ente Nazionale Cinofilia Italiana) were assigned for the study. Puppies were individually identified as reported by Fusi et al. (2018) and monitored for health and normal development until the age of 60 days; bodyweight was measured at 30 and 60 days. For the entire period elapsing between birth and 60 days of age, according to the breeder routine management, the puppies were housed indoors, on rubber mats, so that claw wearing was avoided. Bitches were also monitored for normal postpartum and lactation, and maternal age was recorded. Written informed consent was signed by the breeder to allow the anesthetic and surgical procedure, and to permit the collection of the tips of claws from the puppies they owned for these research purposes.

2.2. Toe claw collection

To standardize the method, in all the cases toe claws were always collected by the same researcher, from all the digits, with a total amount of at least 2 mg being obtained, as determined immediately after collection. Within 12 hours of birth, the tip of each toe claw was clipped and the toe claws from each puppy were pooled for sample evaluations and were stored in an individual paper

envelope, at room temperature, until analysis, as reported by Fusi et al. (2018). At 30 and 60 days of age, the re-growth tissue of the toe claws was clipped and collected; this part of the toe claw tissue was recognizable because it was lighter in color and smaller is size than the toe claw tissue that was present at birth. At each time of collection of toe claw tissue, the toe claws were cut at about 1 mm from the vascular vessel, to avoid bleeding and pain for the animal. The samples collected at 30 and 60 days of age, therefore, also contained a small amount (about 1 mm) of toe claw tissue that was present at the time of the birth or at 30 days post-birth of the puppies, respectively. After the collection of the toe claw tissues, the samples from each puppy were pooled, labeled and stored using procedures that were previously described by Fusi et al. (2018).

2.3. Hormone analysis

Toe claws were cut into small pieces (1-2mm) and washed in 3 mL isopropanol to minimize the risk of extracting steroid hormones from the outer part of the claws, and to ensure the removal of any steroids or other contaminants from the surface of the claw tissues. The samples were extracted in a glass vial using methanol for 16 hours at 37 °C. The solvents in the vial were subsequently evaporated to dryness at 37 °C in an airstream suction hood. The remaining residue was dissolved in phosphate buffered saline (PBS), 0.05 M, pH 7.5. All the samples were freeze dried using procedures reported by Comin et al. (2014), and the dry weights were calculated. The concentrations of 17β-estradiol and testosterone were quantified using a solid-phase microtiter radioimmunoassay, developed in the laboratory in which the research for the present study was conducted using procedures that have been previously described (Comin et al., 2014; Veronesi et al., 2015). The rabbit 17β-estradiol and testosterone antibodies used were obtained from Analytical Antibodies (Bologna, Italy).

There were quality control evaluations for 17β-estradiol and testosterone quantifications. For 17β-estradiol: the total counts were 6,500 cpm; %NSB/total 2.2%; % maximum binding/total 25%; the 20%, 50%, 80% intercepts were 392, 160 and 58 pg/ml, respectively.

For testosterone: the total counts were 9,000 cpm; %NSB/total 1.7%; % maximum binding/total 30%; the 20%, 50%, 80% intercepts were 1,200, 300 and 80 pg/ml, respectively.

The cross-reactivities of the anti-17β-estradiol antibody with other steroids were as follows: 17β-estradiol, 100%; estrone, 2.5%; estriol, 0.12%; 17β-estradiol-(B—D-glucuronide), 0.04%; 17β-estradiol-3-sulfate 0.012%; DHEA, 0.007%; 17α-estradiol, <0.004%; progesterone, <0.004%; testosterone, <0.004%; androstenedione, <0.004%; estrone-3-sulfate, <0.004%. The cross-reactivities of the anti-testosterone antibody with other steroids were as follows: testosterone, 100%; 5α-dihydrotestosterone, 43.2%; 5α-androstanedione, 33.1%; 5β-androstanedione, 11.4%; 5α-

androstan-3α,17β-diol, 9.4%; androstenedione, 0.4%; testosterone 17 β-glucuronide, 0.09%; progesterone, DHEA, 17β-estradiol, androsterone-3-glucuronide, 0.01%; androsterone-3-glucuronide, 0.006%; cortisol, <0.001%. The intra- and inter-assay precision values were determined by analyzing 20 replicates of sample controls in a single assay, and in assays performed on different days, respectively. The intra- and inter-assay coefficients of variation were 4.1% and 11.1%, 4.2% and 12.3% for 17β-estradiol and testosterone, respectively. The sensitivities of the assays were 0.77 and 0.33 pg/well for 17β-estradiol and testosterone, respectively. Parallelism for serial dilutions of pooled claw extracts against the 17β-estradiol and testosterone standard curve were described by the equations $y = 0.9683x + 0.5601$, $y = 0.9828x + 0.4672$ for 17β-estradiol (E2) and testosterone (T), respectively.

2.4. Statistical Analysis

Normal distribution of data was demonstrated using the Shapiro-Wilk test. The ANCOVA test was used to evaluate the effects of fixed factors, such as the sampling time (birth, 30 and 60 days of age) and puppy sex (male or female), and by using covariates, such as maternal age, litter size, bodyweight at birth, 30 and 60 days, Apgar score, and sex steroid concentrations in toe claws for the analyses. The Tukey *post-hoc* test was used to determine if there was an effect of each sampling time on hormonal concentrations from toe claws. The possible effect of the litter on E2 or T concentrations in toe claws was assessed using the Kruskal-Wallis test. The litter effect was not included in the Ancova model to avoid excessive spread of the data marginalizing the power of the statistical analysis. The Kruskal-Wallis test, a more robust statistical method, therefore, was used to assess the possible effect of the litter on steroid concentrations in toe claws. Statistical significance was set at $P<0.05$ (JASP®, ver 9 for Windows platform).

3. Results

3.1. Clinical findings

From the nine bitches assigned for elective CS, a total of 91 puppies were born. Two puppies of two different litters were stillborn and excluded from the study. There, therefore, were toe claw tissues collected to conduct the present study from 89 puppies (46 male and 43 female) of normal viability (Apgar score $\geqslant 7$), normal weight, and without malformations or physical defects.

In the following 60 days after CS during which there were observations, all of the bitches had normal postpartum and lactational characteristics, and all puppies had a normal developmental and growth pattern. As a result, toe claw tissues were collected from all 89 puppies based on the study

designed. The primary data from maternal and puppy evaluations, grouped according to puppy sex, are summarized in Table 1.

3.2. Sex steroid concentrations in toe claws

Toe claw collection was possible in all cases with a sufficient sample amount (about 2-4 mg, depending on the age of the puppy: the greater the age, the larger the sample amount collected) to allow analysis of the toe claw tissues that were collected at the scheduled sampling times. Toe claw growth rate was variable, but always ranged between 2 and 3 mm/claw. The results with use of the ANCOVA indicated there was an effect of sampling time. With use of the Tukey *post-hoc* test, results indicated there a were decreases in concentrations of both hormones from birth to 30 and to 60 days of age, without subsequent changes in concentrations between 30 and 60 days of age. Data on the E2 and T concentrations in the toe claws of the 89 puppies (mean ± SD) at birth, 30, and 60 days of age, are reported in Table 2.

Sex of the puppy was positively (P<0.001) related to T concentration in toe claws, and there was an interaction between puppy sex and sampling time (P<0.01) on T concentrations (Table 3). Among the covariates, the Apgar score was also positively (P<0.001) associated with T concentration in toe claws at birth (Table 4). Additionally, the puppy body weight was also positively (P<0.05) associated with T concentration in toe claws, with an interaction among puppy sex, bodyweight and sampling time (P<0.05) on T concentration in claws (Table 5).

Maternal age and litter size did not have effects on T concentrations in toe claws, however, there was a litter effect (P<0.01) on concentrations of both hormones. Except for sampling time and "litter effect", E2 concentrations in toe claws were not affected by the other variables evaluated in this study.

4. Discussion

In the present study, only puppies with a single breeder were included in the study to avoid the possible effects of management differences (i.e., feeding of the bitches and puppies) on the growth of the puppies. By using animals from only one breeder means, the present results must be considered as only representative of a selected population.

In human growth rate of digit nail is about 3 mm/month (de Berker et al., 2007), while the growth rate in dogs has never, to the best of the author's knowledge, been investigated, and is affected by the amount of walking and the environment (e.g., surface area on which the puppies are housed). In newborn dogs, the problem of puppy consumption of toe claw tissue is minimal, because puppies are housed indoors for almost the entire period between birth and time of sale (60 days of age),

236 avoiding consumption of the toe claws and allowing the repeated collection of the re-growth tissues
237 of claws (about 2-3 mm every 30 days in the present study) at the designated times in the study
238 protocol (Fusi et al., 2018).
239 Although only the re-growth of toe claw tissues was collected at 30 and 60 days of age, at each
240 sampling the toe claws were cut about 1 mm from the vascular vessel, so that at 30 and 60 days of
241 age a small amount of the toe claw tissues that was present at the previous collections was actually a
242 component of the tissues collected. The steroid concentrations quantified at 30 and 60 days of age,
243 therefore, should be considered to be minimally affected by the previous tissue accumulation: from
244 birth for samples collected at 30 days of age, and from 30 to 60 days of age for samples collected at
245 60 days of age.
246 Sex steroids, such as E2 and T, were detectable in toe claws at birth, and again at 30 and 60
247 days after birth, even if hormonal concentrations decreased markedly from birth to 30 and 60 days of
248 age. There was a marked decrease of hormonal concentrations at 30 and 60 days of age as compared
249 to that present at birth. Because the hormone quantifications occurred in the toe claws collected at
250 birth, the concentrations are indicative of the accumulation from the time of the first appearance of
251 the claw during fetal development to the time of birth (it was anecdotally reported that, in dogs, toe
252 claws are detectable at about 30 days of pregnancy; Veronesi et al., 2015). It, therefore, was
253 emphasized, that the E2 and T concentrations present at the time of birth must be attributed to the
254 prenatal deposition of the sex steroids into the toe claws of the fetuses. Similar to the data reported
255 for horses (Dhakal et al., 2012) when there were sex steroid quantifications in matrices other than
256 claw tissues, in the present study, sex steroid concentrations in the toe claws also decreased
257 substantially after birth to 30 days subsequent to birth. As was suggested for horses, during the
258 neonatal and early pediatric period of dogs, the gonads most likely do not have a pivotal function in
259 the processes of post-natal development and growth until puberty approaches.
260 Regarding the results in the present study from the steroid accumulation in the fetal toe claws,
261 it remains difficult to discern the source of steroid production. Although it has been reported that the
262 fetal gonads are responsible for significant sex steroid (especially testosterone) production in other
263 species, the authors are not aware of similar evidence for dogs. The relatively larger quantities, as
264 compared to 30 and 60 days post-birth, of sex steroids accumulated in the fetal toe claws could be the
265 result of fetal sources of production, maternal sources of production, or both, even if the maternal
266 source could be the main contributor, because of the large amount of sex hormones produced by the
267 extraembryonic membranes in most mammals. The relation between T concentrations in toe claws,
268 and puppy sex, with greater T concentrations in male compared to female puppies, seems to support
269 the hypothesis of a fetal T secretion, as reported in humans (Van de Beek et al., 2004; O'Connor and

Barrett, 2014) and other animals, such as the horse (Dhakal et al., 2012). Results of studies with humans and horses indicate the greater prenatal T concentrations in males than females at the time of birth are the result of the earlier fetal development of testicles than ovaries. The results from the present study indicate this explanation also applies to dogs. Furthermore, the fetal secretion of T in dogs could also be supported by the characteristic of the dog to bear both males and females within a litter.

Regarding the function of these sex steroids before birth, prenatal sex steroids may have function not only for fetal development, but also in the complex tissue developmental programming of several organs and systems in dogs. The results of the present study support this possibility because of the effect of covariates, such as the Apgar score and bodyweight on T toe claw concentrations. The highly significant association between T concentrations in toe claws and the Apgar score is interesting, but it must be considered cautiously. All the puppies were viable in the present study, as indicated by the Apgar score $\geqslant 7$. The marked differences in the T concentration in toe claws among the puppies with the 7 to 10 Apgar scores leads for speculation that there are possible positive effects of androgens on puppy viability around the time of birth. Furthermore, results of previous studies in humans and dogs have never been reported where there was assessment of neonatal viability at birth, Apgar scores, and androgen concentrations. Inconsistent with this possibility, in one study (Stevenson et al., 2000), it was reported that, in comparison with females, male babies have a greater risk for a lesser Apgar score when determinations were made 1 and 5 minutes after birth. Furthermore, the results from the present study are drawn from a relatively small number of subjects, especially when these are grouped according to Apgar score. It is also interesting to note the effect of T on the bodyweight of puppies. In the present study, prenatal testosterone concentrations were positively associated with a larger bodyweight at 30 and 60 days of age in males, in comparison with females, a finding that is difficult to explain. It could be hypothesized that androgens have a function in fetal programming of post-natal weight gain, even though Manikkam et al. (2004) reported a different postnatal bodyweight gain in female and male lambs when there were prenatal treatments with testosterone. There is no awareness of studies from which there are reports of the association between physiologic T fetal concentrations and a larger postnatal bodyweight.

Unlike results obtained from amniotic fluid in humans (Van de Beek et al., 2004), in the present study E2 concentrations in toe claws did not differ as a result of sex of the puppies, indicating there is a lesser action of this steroid as compared with T on fetal development at the stages when there were assessments in the present study. This finding, however, is interesting and requires further investigation, maybe by evaluating sex steroid concentrations in other matrices, such as amniotic

fluid.

There were large standard deviations for concentrations of both sex steroids at all sampling times in the present study. Beside the possible inter-individual variation, the so-called "litter effect" may be an important consideration because it is possible that these large standard deviations could also be related, at least partially, to a maternal effect on accumulation of both E2 and T in the fetal toe claws of puppies.

5. Conclusions

In conclusion, the results of the present study further confirmed the usefulness of toe claws as a possible matrix for the retrospective study of hormonal changes in perinatology of dogs. The results from the present study indicate E2 and T concentrations are greater at birth compared with that at 30 and 60 days subsequent to birth. These fetal steroids, therefore, could have prenatal actions on fetal development. Similar to results from other species, in dogs, there are greater prenatal T concentrations in male than female puppies at birth. Furthermore, and surprisingly, there is an interaction with male puppies having a larger bodyweight also having greater T concentrations during the first 60 days of age. There were also greater Apgar scores, indicating greater fetal viability, associated with T concentrations at birth independently of the sex of the puppy.

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Table 1

Descriptive maternal (*n* =9) and puppy (*n* =89) clinical findings grouped according to puppy sex (BW: body weight)

Puppies (*n* =89)	Maternal age (years)	Litter-size (*n*)	BW birth (g)	BW 30 days (g)	BW 60 days (g)	Apgar score
Male (*n* =46)	3.9 ± 0.9	6.7 ± 1.8	428 ± 87	3460 ± 425.8	7958 ± 985.7	8.3 ± 0.8
Female (*n* =43)	3.5 ± 0.8	6.3 ± 1.6	404 ± 61.4	2858 ± 292.5	6005 ± 873.4	8.0 ± 0.8

Table 2

E2 and T concentrations (mean ± SD) in toe claws at birth, 30 and 60 days of age in the 89 puppies

	Birth	30 days	60 days
E2 (pg/mg)	10 ± 6.3^{a}	2.7 ± 1.3^{b}	2.1 ± 1^{b}
T (pg/mg)	9.9 ± 6^{a}	4.1 ± 2.6^{b}	2.5 ± 1.2^{b}

[a,b]Different superscripts indicate differences $P<0.001$

382 **Table 3**

383 Concentrations (mean ± SD) of T in toe claws at birth, 30 and 60 days of age in the 89 puppies grouped

384 according to puppy sex

	Birth T (pg/mg)	30 days T (pg/mg)	60 days T (pg/mg)
Males (*n* =46)	12.3 ± 6.9	4.6 ± 3	2.5 ± 1.4
Females (*n* =43)	8.2 ± 4.6	3.7 ± 2.1	2.5 ± 0.8

385

386

387 **Table 4**

388 Concentrations (mean ± SD) of T in toe claws at birth of the 89 puppies grouped according to Apgar score

	Apgar score 7 (*n* =17)	Apgar score 8 (*n* =53)	Apgar score 9 (*n* =9)	Apgar score 10 (*n* =9)
T at birth (pg/mg)	7 ± 2.7	7.3 ± 6.4	9 ± 3.6	18.3 ± 7.3

389

390

391

Table 5

Concentrations (mean ± SD) of T in toe claws and body weight at birth, 30 and 60 days of age in the 89 puppies grouped according to puppy sex

	Birth		30 days		60 days	
	Males (*n* =46)	Females (*n* =43)	Males (*n* =46)	Females (*n* =43)	Males (*n* =46)	Females (*n* =43)
BW (g)	428 ± 87	404 ± 61.4	3460 ± 425.8	2858 ± 292.5	7958 ± 985.7	6005 ± 873.4
T (pg/mg)	12.3 ± 6.9	8.2 ± 4.6	4.6 ± 3	3.7 ± 2.1	2.5 ± 1.4	2.5 ± 0.8

Effect of breed body-size on leptin amniotic fluid concentrations at term pregnancy in dogs

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Effect of breed body-size on leptin amniotic fluid concentrations at term pregnancy in dogs

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Abstract

Because of the need to improve the knowledge about canine perinatology, and given the major role of fetal fluids in sustaining the course of pregnancy and fetal development, an in-depth analysis to better understand the role of some hormones in these compartments is essential. Among all, leptin is recognized to play a key role not only on the energetic homeostasis, but also at multiple levels, influencing the control of reproduction, food assumption and metabolism. Even if in humans and other species it is reported the presence of leptin receptors during fetal development, very little is known about the canine species, in which the role of leptin still needs to be fully understood. The present study aimed to assess the amniotic fluid leptin (AFL) concentrations at term pregnancy in healthy dogs, and to evaluate the possible influence played by breed body-size (after assessment of

correlation with maternal bodyweight and placental weight), or other maternal (age, parity, and the so-called "litter effect") and neonatal (gender, birth weight, litter size) parameters on AFL concentrations, analyzed by ELISA test. The study was performed on 90 healthy, viable and normal weighted puppies, 39 small-sized (adult body weight < 10 kg) and 51 large-sized (adult body weight > 25 kg), born by 29 purebred, healthy bitches, submitted to elective Caesarean section because of breed-related or individual high risk for dystocia. The results showed that the mean AFL concentration in the small-sized puppies was significantly ($p<0.05$) higher in comparison to large-sized puppies (867.48 vs 698.42 pg/ml), while all the other studied parameters did not show to influence AFL concentrations. In conclusions, the present study showed significant higher at term AFL concentrations in small-sized as compared to large-sized breeds, suggesting an influence of breed body-size on fetal metabolism, as previously reported for NEFA and IGF-I.

Key words: dog, breed body-size, term pregnancy, amniotic fluid, leptin

1. Introduction

The study of canine perinatology has recently raised increased interest under a two-fold perspective: on one hand there is the need to deepen the knowledge about the canine perinatal physiology, on the other hand the availability of reference physiologic data is necessary for the further detection of possible markers for the diagnosis of diseases or abnormalities. One of the limitations to the study of canine perinatology is addressed to the difficulty to perform biological samples collection before and even after birth. This limitation represents also a concern from an animal welfare standpoint, especially when blood sampling is considered. Moreover, in dogs, although amniocentesis can be used to collect amniotic fluids in late pregnancy, it was reported that it could be difficult to achieve, because puncturing the amniotic sac is possible if the bitch stays calm and in a dorsal recumbent

position [1]. However, several studies demonstrated that, in dogs, amniotic fluid could be safely collected during Caesarean section to define the fetal fluid composition at the time of birth, providing information about the normal composition [2-5] and preliminary results on differences in pathological newborns [4,5].

Among the study performed on canine fetal fluids composition, [2] investigated the IGF-I and NEFA fetal fluids concentrations, and reported a breed body-size association between lower IGF-I and higher NEFA concentrations in small- as compared to large-sized breeds, raising the question about the possible influence of breed-size on some metabolic amniotic components in dogs.

Beside IGF-I and NEFA, also leptin plays a key role in the energetic homeostasis. Leptin, is a protein mainly produced by white adipose tissue [6] with lesser quantities produced also from other tissues, that acts at multiple levels with pleiotropic roles in the control of reproduction, food assumption, energy expenditure, and metabolism [7,8]. The role of leptin in the fetus did not receive an extensive investigation, although some studies described the role of leptin, or the presence for leptin receptors, in different organs in humans and some animals, but not in the dog, during fetal development [9-11]. Leptin was also reported to be involved in the process of growth and development, so that alterations in leptin were reported to be considered as indications of disease risk [12]. Canine leptin plasma concentrations have been investigated by Ishioka et al [13], in relation to body condition score, age, gender and breed. Those authors found a positive correlation between plasma leptin concentrations and body condition score, but no effects of age, gender and breed, demonstrating the role of leptin as a marker for adiposity in dogs. Leptin secretion is modified in obese women and overweight pregnant bitches were reported to be more prone to dystocia [14]. Recently [15], leptin circulating concentrations have been described along dog pregnancy and lactation, highlighting a first increase from 0 to 45 days of pregnancy, with further decrease at 60 days of pregnancy and a subsequent increase in the two months of lactation. Balogh et al [16] studied the gene expression of leptin and leptin receptors in the uterus and placenta of

77 bitches at different stages of pregnancy, and concluded that, in the canine species, the uterus and
78 placenta are source for leptin production but also target organs of its autocrine/paracrine actions
79 [16].

80 To the authors knowledge no studies on canine amniotic leptin concentrations have been, so far,
81 reported. However, amniotic fluid leptin was investigated in other species. In humans, amniotic
82 fluid leptin concentrations were reported to originate by amniotic cells, but also from fetal adipose
83 tissue and subsequent transfer to the fetal circulation [17,18]. The maternal concurrence to amniotic
84 fluid leptin concentrations is considered scarce in humans, because of its high molecular weight,
85 and the consequent process of leptin compartmentalization [17,19]. When the association between
86 amniotic fluid leptin concentrations and some fetal and maternal parameters have been investigated
87 in humans, mid trimester amniotic fluid leptin levels resulted positively correlated to maternal body
88 mass index, and negatively correlated to parity [19], while no associations were found between
89 amniotic fluid leptin concentrations and placental or birth weights, maternal age, ethnicity, fetal
90 gender and pregnancy outcome [19].
91 Previous studies showed associations of some amniotic fluid biochemical parameters [5] and
92 amniotic fluid IGF-I and NEFA [2] with canine breed body-size, suggesting that breed body-size
93 could have an influence on homeostasis. Aware that many parameters can specifically influence the
94 energetic homeostasis, the present study focused on the measurement of amniotic leptin
95 concentrations in non-obese dogs belonging to different body-size, in order to provide an additional,
96 although limited, information about the complex contribution to energetic homeostasis in canine
97 fetuses/neonates. Because of the reported possible impact played by other parameters on amniotic
98 leptin concentrations, beside the effect of breed body-size, the possible effect of some other
99 maternal (age, parity, and the so-called “litter effect”) and neonatal (gender, birth weight, litter size)
100 on amniotic leptin concentrations was also assessed. The “litter effect” is considered as a possible
101 parameter referring variation among litters: (i.e. differences between puppies belonging to different
102 bitches).

2. Material and Methods

2.1 Animals

The study was performed on 29 purebred, healthy bitches, aged 2-7 years, 8 primiparous and 21 pluriparous (2-5 parturitions). According to breed body-size, 15 bitches were small-sized (body weight <10 kg)(7 Chihuahua, 4 Spitz, 2 Miniature Bull Terrier, 1 Maltese, and 1 Bouledogue Francaise), and 14 were large-sized (body weight >25 kg)(4 Maremma, 2 Bernese Mountain, 2 Labrador Retriever, 2 German Shepherd, 2 Newfoundland, 1 Hovawart, and 1 Saint Bernard).

All the bitches were healthy, regularly vaccinated and dewormed. They showed a pre-pregnancy body condition score of 3/5, and were fed twice-a-day during pregnancy with a commercial diet for dogs, meeting the metabolic energy needs on the base of the nutritional guide-lines recommendations for dogs and cats. The amount of food was calculated for each bitch, on the base of breed body-size and body weight, and modified along pregnancy according to the number of fetuses. Food assumption was regularly checked twice-a-day along the entire pregnancy. At the end of pregnancy, none of the bitches showed body weight increases exceeding 30% of the pre-pregnancy body weight, so that all of them were considered as non-obese dogs.

They were clinically monitored from the time of mating/artificial insemination, along pregnancy, until whelping. Considering the breed-related or individual high risk for dystocia, all the bitches were submitted to elective Caesarean section at the estimated date of parturition, for the health of mothers and puppies. The date of parturition was calculated according to previous reports [2-5], taking in consideration the blood progesterone concentrations at the sole mating/AI, the embryonic/fetal biometry, the clinical and ultrasonographic maternal and fetal monitoring at term, and the blood progesterone concentrations at impending parturition. Caesarean section was performed using an anesthesia protocol, aimed to minimize the possible side-effects on newborn puppies' viability, as previously reported by [2].

Two expert neonatologists took care of the puppies immediately after uterine extraction, providing first assistance and evaluating neonatal viability by measuring the Apgar score within 5 minutes after birth [20], assessing the absence of gross malformations or physical defects, recording newborn gender and measuring birth weight before nursing. Birth weights within the breed reference range reported by the Italian Kennel Club (Ente Nazionale della Cinofilia Italiana) were considered as normal. According to Veronesi et al [20], only normal viable (Apgar score ≥7), and normal weighed newborns were considered. Individual placentae were weighted according to [21].

Before surgery the owners signed an informed consent to allow anesthesia and surgery, but also to specifically allow the collection of amniotic fluid and its use for research purposes.

2.2 Amniotic fluids collection

At fetal extraction, one person was dedicated to amniotic fluid collection from each amniotic sac, as previously reported [2]. Individual amniotic fluid samples were immediately centrifuged at room temperature at 1000xg for 10 minutes, and the supernatant placed in plastic vials and frozen at -20° C until leptin analysis.

2.3 Leptin amniotic fluid analysis

At the time of analysis, amniotic fluids were centrifuged for 15 minutes at 1000xg to remove debris. The concentration of leptin in the amniotic fluid samples was analyzed by competitive canine leptin ELISA kit (MyBioSource, San Diego, USA) utilizing a polyclonal anti-leptin antibody and a leptin-HRP conjugate. The ELISA was performed according to manufacturer's instructions. The sensitivity of the leptin ELISA was 0.1 ng/ml. The intra-assay and inter-assay coefficients of variation were <10% and <10%, respectively. The relationships among amniotic fluid sample and the standard curve, determined through linear regressions, was linear with correlation coefficients of r=0.99. The empirical regression line is given by the equation y=0.79+1.01x.

2.4 Statistical analysis

Firstly, we calculate two regression functions, between maternal body weight and breed body-size and between placental weight and breed body-size, respectively, in order to corroborate the choice of breed body-size as bitches grouping factor. Since the breed body-size is a qualitative covariate, it was incorporated into the regression using a dummy variable X (i.e. X=0 for the large breed body-size and X=1 for the small breed body-size). Then, we interpreted the coefficient of determination R squared, which ranges from 0 and 1 with values closer to 1 indicating a better fit to the regression model. Moreover, consider that in case of a simple linear regression R squared corresponds to the squared Pearson correlation coefficient. Afterwards, in order to verify if there was a difference in the amniotic fluid leptin concentrations in dependence of a breed body-size, a welch-test was conducted for small-sized against large-sized dogs, under assumption of normality and of heteroscedasticity (i.e., different variances in the two groups) of the leptin concentrations. We can easily assume data normality due to the central limit theorem and the fact that our samples, containing more than 30 puppies, can therefore be considered large (rule of thumb). Variance heterogeneity may occur naturally; tests allowing heterogeneity are more robust and should be used in routine. Statistical significance was set as usual at $p<0.05$.

To assess if other parameters could also be responsible for the amniotic fluid leptin concentrations, a linear mixed model was fit for it, in dependence not only of the breed body-size but also of other maternal (age, parity, "litter effect") and neonatal (gender, birth weight, litter-size) parameters.

All analyses were made by R (version 3.6.0) as reproducible report by the R package Knitr and could be made available upon request.

3. Results

3.1 Clinical findings and bitches grouping

A total of 90 healthy, viable and normal weighted puppies, 39 small-sized and 51 large-sized, were born; litter size ranged between 1 and 5 in small-sized bitches, and between 1 and 8 in the large-

sized ones. Based on gender, 40 puppies (44%) were females, and 50 (56%) were males. Mean ± SD birth weight was 191±77.57 g in the small-sized, and 583±132.83 g in the large-sized newborns.

In small-sized bitches the mean ± SD maternal body weight was 5.02±2.93 kg (range: 2-9.3 kg), while in large-sized bitches it was 44.0±10.46 kg (range: 29-62 kg). Mean ± SD placental weight in small-sized bitches was 25.3±7.26 g (range: 19.1-45.8 g), while in large-sized bitches it was 64.3±10.34 g (range: 47.7-80 g). The statistical analysis showed a strong correlation between maternal body weight and breed body-size (Rsquared=0.85, $p<0.001$), as well as of placental weight and breed body-size (Rsquared=0.76, $p<0.001$), corroborating the usefulness of breed body-size for bitches grouping, thanks to its easier and more practical recording in comparison to body weight and/or placental weight measurement.

3.2 Amniotic fluid leptin concentrations

The distribution of leptin amniotic fluid concentrations in the 39 small- and in the 51 large-sized puppies is reported in Figure 1.

The mean amniotic fluid leptin concentrations was 867.48 pg/ml in the small-sized puppies and 698.42 pg/ml in the large-sized newborns, with standard deviations of 209.32 pg/ml and 161.71 pg/ml, respectively. Correspondingly, the 95% confidence interval was 867.48 ± 65.69 pg/ml in the small-sized puppies and 698.42 ± 44.38 pg/ml in the large-sized newborns. According to the welch-test, this is a significantly different concentrations ($p<0.05$).

Figure 1 – Distribution of leptin amniotic fluid concentrations in the 39 small- and in the 51 large-sized puppies rendered by boxplots. Each point represents one puppy.

PUT HERE FIG 1

On the other side, the fitted mixed model for amniotic fluid leptin concentrations in dependence of other parameters did not show significant influence played by any of the other maternal (age, parity, "litter effect") and neonatal parameters (newborn gender, birth weight, and litter size).

4. Discussion

This study showed that leptin is detectable in canine amniotic fluid collected at term of pregnancy, with concentrations very similar to those reported in humans by >33 weeks of gestation (median value: 519 pg/ml, range: 380-761 pg/ml) [22]. Based on a previous study in which differences between small- and large-sized canine breeds in IGF-I and NEFA amniotic fluid concentrations were reported [2], the present study investigated the possible influence of breed body-size on leptin amniotic fluid concentrations, as a result of the role of canine breed body size on metabolic hormone concentrations in amniotic fluid collected at term of pregnancy. Thanks to the strong correlation between breed body-size and bitches body weight and placental weight, breed body size was demonstrated to be a useful parameter to assess the effect on leptin amniotic concentrations. Breed body size represent a more practical and easier to be recorded parameter instead of measuring maternal body weight and placental body weight, under normal conditions.

The dog is a unique species in which hundreds of different breeds are included, with body size ranging between the smallest Chihuahua (body weight max 3 kg), to the largest Saint Bernard (body weight up to 120 kg). This implies a diverse metabolism among breed sizes, that can be displayed at different age or at different physiologic conditions. A recent study by Cardinali et al [15], showed a low correlation between pregnant dogs' plasma leptin and body weight, but the evaluation of a breed influence was not possible, because all the bitches belonged to the same (Bloodhound), large-sized breed. In humans, amniotic fluid leptin mid-trimester concentrations were positively correlated to maternal body mass index, and negatively correlated to maternal parity, but not related to fetal gender, birth weight or pregnancy outcome [19]. In the present study, leptin amniotic fluid concentrations measured at term pregnancy were not significantly influenced by maternal age,

parity, “litter effect”, fetal gender, birth weight, and litter size. The different result concerning the influence of maternal parity in humans, as compared to dogs, may be related to the different characteristics of the placenta in the two species. Unfortunately, a comparison with similar studies on dogs is not possible, also considering leptin maternal plasma concentrations. In fact, in the study from Cardinali et al [15], the possible effect of parity on leptin maternal plasma concentrations was not assessed. It is clear that a weakness of the present study is the lacking of maternal blood leptin concentrations measurement at parturition. In fact, although the bitches were vein cannulated for anesthesia, most of the owners, especially those of small-sized bitches, did not allow for blood sampling for research purposes. Measuring maternal blood leptin concentrations at the time of parturition would be interesting to evaluate the possible correlation between maternal and offspring leptin concentrations.

Similar to what reported in humans, also in dogs it could be interesting to investigate the gestational age-related changes in amniotic fluid leptin concentrations, even if, as stated before, amniocentesis remains a procedure that could be difficult to achieve. However, Balogh et al [16] reported the different expression of leptin and leptin receptors in the canine uterus and placenta during different stages of pregnancy, and suggested the possible regulatory role of leptin around the time of parturition.

It is interesting to note the absence of significant associations between amniotic fluid leptin concentrations and puppy’s birth weight. In fact, although the mean birth weight was different between small- and large-sized breed’s puppies, amniotic fluid leptin concentrations were not significantly influenced by birth weight. It must be highlighted that, in all the puppies, the birth weight was in the normal ranges reported for the breed, and no under- or over-weighted newborns were born. In humans, the relation between higher leptin concentrations in maternal serum, fetal serum, amniotic fluid or placenta, and growth perturbation, have been reported, even if it was suggested that the leptin levels in different biological specimens could represent diverse markers for

249 interconnected, but different, process of development along pregnancy [22]. Some authors found a
250 positive correlation between cord blood leptin concentrations and body weight in all the human
251 neonates studied [23] or only in the prematures [24], but others [22] did not find an association
252 between amniotic fluid leptin concentrations and birth weight, with amniotic fluid leptin
253 concentrations only marginally higher in small for gestational age newborns.

254 Some authors [25] reported higher human amniotic fluid leptin concentrations in female than in
255 male fetuses, suggesting a possible sexual dimorphism as an additional regulatory growth
256 mechanism. However, similar to the present study results, other authors did not find significant
257 differences in human amniotic fluid [22] and in cord blood [26,27] leptin concentrations between
258 females and males, arising the doubt about a different, gender-related, body fat amount in
259 newborns.

260 It was interesting to note that the "litter-effect", did not show to influence amniotic leptin
261 concentrations, suggesting that the differences among litters in amniotic fluid leptin concentrations
262 unlikely could concur to explain the wide standard deviations found in the present study.

263 **5. Conclusions**

264 In conclusions the present study showed that leptin is detectable in canine amniotic fluids collected
265 at term, from healthy purebred puppies, born by non-obese bitches. Significant higher
266 concentrations were detected in small-sized as compared to large-sized breeds, suggesting an
267 influence of breed body-size on fetal metabolism.

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1250
1000
750
500
Amniotic Fluid Leptin concentration (pg/ml)
Small
Large
Breed body−size

Project line 3
PUBERTY

Preface

This project line involved the study of the longest period investigated in the present PhD project and, likely the line about pregnancy and post-partum, it was also affected by the Covid-19 emergency.
Given that this study was aimed to investigate the hormonal changes occurring on coat and claws from weaning to puberty attainment in dogs and cats, the total time of study was estimated to last for 4-20 months. Although also this study started at the first year of the PhD course, the conclusion of the coat and claws samples, monthly collected, was completed in 12 dogs, but the scheduled analysis at the laboratory were postponed to Autumn 2020, preventing the use of the results for this thesis, because of the Covid-19 emergency. Although it was initially planned to perform the same study also in cats, the cat breeders demonstrated themselves less compliant than dog breeders and did not allow to enroll cats for this long-lasting study. Therefore, the study on cats was shifted to a more feasible, acceptable by cats' owners, investigation in which a single coat and dewclaws sample was collected during routine clinical examination of cats of known reproductive status (prepubertal, pubertal, gonadectomized), and analyzed for the two main sexual steroids: 17β-estradiol (E2) and testosterone (T). Other hormones, such as progesterone, C and DHEA-S were planned to be assessed, but the laboratory could only provide results about E2 and T as preliminary results. A similar study was also performed in dogs, to assess the reliability of a single collection of coat and claws for the hormonal measurement in males and females of known reproductive status. In this case, also, unfortunately the limitation to the laboratory work dictated by the COVID-19 emergency rules allowed the analysis of only some of the scheduled hormones measured and only in coat. Therefore, the results presented in the following two studies must be considered only preliminary.
In the first study, performed on 55 cats of known reproductive status (prepubertal, pubertal, gonadectomized), coat and dewclaws were collected only once and the concentrations of 17β-estradiol (E2) and testosterone (T) were assessed. The data obtained were then grouped in relation to sex and reproductive status. The results showed that both matrices were useful for hormonal measurements. However, some statistical significances were found only for the coat, highlighting that the reliability of dewclaws for this kind of studies in cats must receive further investigations. The study showed that the concentrations of T in coat was significantly different between prepubertal males and females and between gonadectomized male and females. Only the concentrations of T in coat were different between pubertal and prepubertal male cats. These results provide useful information for the further investigations about the use of these matrices, collected only once, to distinguish prepubertal, pubertal

and gonadectomized subjects within a sex, in the domestic cat. However, further implementation (higher number of cases, equal distribution of cases within each sex and within each reproductive status, better definition of each reproductive status, wider hormonal analyses) are requested for a more accurate understanding of the results.

In the second study, 40 dogs of known reproductive status (prepubertal, pubertal, gonadectomized), were submitted to a single coat collection used for the measurement of 17β-estradiol (E2), testosterone (T), cortisol (C) and dehydroepiandrosterone sulfate, indicated as DHEA(S). The results confirmed the usefulness of the dogs' coat as a matrix for the measurement of retrospective and long-term accumulation of hormones, even though some weaknesses related to the single sampling arose and deserve further investigations. Therefore, the obtained results must be considered only preliminary and should be cautiously interpreted.

In prepubertal and pubertal dogs, only T concentrations in coat were significantly higher in males than females. In pubertal dogs, DHEA(S) and C coat concentrations were higher in males than females. Although higher T and DHEA(S) in males are not surprising, the higher coat C concentrations in males than females seems to suggest that the pubertal state could be more stressful for males.

The assessment of possible differences in the long-term concentrations of hormones in the coat, among the three reproductive statuses and within each sex, showed a significantly lower DHEA(S) concentration in pubertal than prepubertal and gonadectomized female dogs. In males, T concentrations in coat were higher in pubertal than prepubertal dogs, and C concentrations in coat were higher in pubertal than prepubertal and gonadectomized dogs. Data seem to suggest that the pubertal status in male dogs entails not only the expectable higher exposition to T in comparison to the prepubertal stage, but also that pubertal status leads to a higher activation of the HPA axis and, in turn, C secretion that accumulates in the coat. In conclusion, the results of the present study showed that some interesting differences between the two sexes in different reproductive statuses, as well differences between reproductive statuses within each sex, were found. However, the single collection of coat provides information affected by several factors that need to be better investigated.

COAT AND DEWCLAWS FOR 17β-ESTRADIOL AND TESTOSTERONE CONCENTRATIONS MEASUREMENT AND REPRODUCTIVE STATUS DEFINITION IN CATS: PRELIMINARY RESULTS

Introduction

Puberty represents a milestone in both humans and animals, marking the beginning of reproduction after a complex interaction and coordination of physiologic processes leading, in a gradual manner, to the functionality of the genital system (Vercellini et al., 2018; Alotaibi, 2019). Generally speaking, puberty is coupled with physical, behavioral and especially hormonal changes culminating in spermatogenesis and ejaculation in the male, and in first estrus and onset of ovarian activity in the female (Sjaastad et al., 2013).

In the domestic cat (*Felis silvestris catus*), like other species, puberty is under the control of the Hypothalamic-Pituitary-Gonadal axis (HPG) axis, interacting with the external environment and with the nervous system (Sjaastad et al., 2013). Usually, in the cat, puberty occurs within a range of age of 4-21 months (Traas, 2015), and this variability can be addressed to several factors, including genetics, and in turn, the breed and family line (Verstegen, 1998; Griffin, 2002). Another typical characteristic of cat reproduction, and with a major role in puberty onset, is seasonality. Cats, indeed, are long-daylight breeders, positively influenced by the lengthening of the daylight, that starts, in the northern hemisphere, in late January-February (Hurni, 1981). According to the season of birth, female cats born in summer-autumn will become pubertal in the next spring, at the age of about 5-6 months, while cats born in spring will reach puberty at the age of about one year (Tsutsui et al., 2004). Nutrition is also important for puberty attainment, especially for females (Sjaastad et al., 2013), as well as social stimuli, such as the presence of a female in estrus or of a male, able to anticipate the onset of puberty (Griffin, 2002). The first estrus, in most females, occurs when they have reached a bodyweight of 2.3-2.5 kg, corresponding to about 80% of the adult bodyweight, and is usually seen at about 6-9 months of age (England, 2010). In female cats, estrus is detectable by the behavioral changes due to the beginning of ovarian activity and estrogen production and secretion (England, 2010). The cat is also an induced-ovulatory species (Verstegen, 1998), so that, without mating, proestrus and estrus phases are followed by the repetition of other proestrus and estrus phases. However, spontaneous ovulation can occur in 35-60% of cats (Tsutsui et al., 2009), and in this case a diestrus phase will follow.

In male cats, the first ejaculation can occur as early as the 7th month of age, while mating can start at about 8-10 months of age, depending on nutrition and season (Traas, 2015). Sexual behavior, however, can be displayed from 8-12 months of age, even if some first sexual behavior signs can be observed as early as the 4th month of age (Christiansen, 1987). The typical pubertal sexual behavior in male cats is urine spraying (Horwitz, 2019). Puberty in male cats is related to the appearance of the penile spines, androgen-dependent anatomic structures (Christiansen, 1987), absent in prepubertal cats and generally disappearing about 5-6 months after orchiectomy. However, in males orchiectomized in adult age, the penile spines can disappear in a slower manner. Some disorders, such as delayed puberty (England, 2009) and silent estrus (England, 2010) are recognized in the female cat, representing an issue for cat breeders. For these disorders, their timely recognition could lead to a prompt investigation and possible resolution.

On the contrary to cats breeding, populations of stray cats represent an issue that have been managed for a long time with gonadectomy through the program "trap, neuter and release". In practice, however, when stray cats are concerned, many of the female presented at the animal welfare organizations are of unknown reproductive status (Morrow et al., 2019), and sometimes males recognized as neutered are unilateral cryptorchid, thus continuing to display sexual behavior. The gold standard method to define the neuter status is the exploratory laparotomy: invasive, costly and with potential risks (Morrow et al., 2019). In alternative to exploratory laparotomy the neuter status of the cat can be assessed by blood estrogens or testosterone analysis. These methods are based on the recognized sharp decrease of sexual steroids already one week after gonadectomy in cats (Martin et al., 2006). Recently, a patient-side analysis of luteinizing hormone concentrations in blood proved to be reliable to detect the unneutered status in female cats (Morrow et al., 2019).

However, also the simple blood collection can be stressful for the cats, so that alternative methods of hormonal investigations are desirable. Therefore, the availability of a simple, noninvasive, tool, able to discern pubertal from prepubertal, and neutered from unneutered cats could be useful.

All the reproductive functions are under major control of testosterone, estrogens and progesterone, mainly produced by the gonads (Sjaastad et al., 2013). In both males and females, sex steroids secretion is controlled by the HPG axis, developing before birth, briefly active immediately after birth and then inactive until puberty (Herbison, 2016). A recent study investigated testosterone (T) and 17β-estradiol (E2) fecal concentrations in male and female kittens from birth to puberty and found significantly higher sexual steroids fecal concentrations in the first 4-5 weeks of age than the following weeks, until puberty (Faya et al., 2013). Interesting, fecal estrogens and testosterone were found to be higher at birth

than during estrus in females, and also as compared to sexually mature intact males. However, the study of puberty attainment in the cat is still largely not investigated, although its deepening is interesting not only because of the need of increasing basic knowledge, but also for the recognition and understanding of disorders related to this complex reproductive milestone.

Sexual steroids, the main hormones secreted by the gonads, can be measured on several matrices, such as blood plasma or serum, saliva, urines, feces, and in some cases, milk (Mesarčová et al., 2017). Other matrices, such as the hair and the nails, recently studied in human and veterinary medicine, have been considered as alternative to those listed above, and proved several advantages in their use (Mesarčová et al., 2017). However, depending on the specific study, all the advantages and disadvantages of each matrix should be thoroughly evaluated for a correct choice. Briefly, blood plasma and serum have been traditionally used for hormonal analyses (Mesarčová et al., 2017). These matrices, however, are collectable by blood sampling, considered invasive and thus not useful as a first-line choice. Moreover, the measurement of hormones on blood provide only punctual information, so that a single measurement is not useful for evaluating hormones secreted in an episodic manner, such as, e.g., testosterone. This aspect is real also when saliva is considered: blood and saliva are useful to detect only acute changes (Mesarčová et al., 2017). Therefore, when long lasting hormonal changes are investigated, the serial repetition of samplings could be necessary (Mesarčová et al., 2017). This contrasts with the need of respecting the animal welfare, even when saliva sampling is concerned. In fact, also saliva samplings need a mild-to-moderate handling (Mesarčová et al., 2017). Urines and feces, instead, can be easily collected without invasiveness (Pineda-Galindo et al., 2017). In contrast to the "punctual" information of blood and saliva, urines depict hormonal concentrations of the last 12-24 hours (Bergamin et al., 2019). However, for these two matrices, the concentrations of hormones can be influenced by several factors, such as changes related to daily and seasonal variability (Schell et al., 2017). Moreover, when not analyzed immediately after collection, all these matrices need to be stored by freezing (Sudsukh et al., 2017; Qian et al., 2018). In this context, when long-term changes of steroid hormones are investigated, hair/coat and nails/claws represent the matrices of first choice (Mesarčová et al., 2017; Voegel et al., 2018), stable at room temperature and storable without the need of freezing (Voegel et al., 2018).

Many studies proved the reliability of hair/coat and nails/claws for the retrospective evaluation of long-term accumulation of hormones in both humans and animals, thanks to the ability to incorporate hormones that remain stable until analysis (Accorsi et al., 2008; Doan et al., 2018; Whitham et al., 2020). Palmeri and coworkers (2000) demonstrated the mechanism by which compounds are

incorporated in the nails, and more recently Higashi and colleagues (2016) showed, in humans, the positive correlation between lipophilicity of a compound and its affinity with the keratin of nails. Therefore, sex steroids such as estrogens and testosterone, reported to stably bind to keratin, could be measured with good results in nails (Higashi et al., 2016). On the other hand, these peculiarities make these matrices not suitable to detect acute hormonal changes (Mack and Fokidis, 2017). Both matrices have the advantage to limit the number of samplings in longitudinal studies, and to be collectable without invasiveness, thus complying the issue of animal welfare.

To the author's knowledge, after a first report by Accorsi and colleagues (2008), hair was used for the analysis of cortisol concentrations in cats with chronic stress due to *Microsporum canis* infection (Galuppi et al., 2013). The authors found that, in the hair collected from the thigh of clinically infected cats, the cortisol concentrations were higher than in the hair of negative cats. The authors reported that the technique was noninvasive and useful, according to both ethical and logistic reasons, and suggested that the analysis of steroid hormones in the hair could be useful in studies requiring monitoring of function for extended period and for assessing the hormonal substrate of the organism (Galuppi et al., 2013). However, when sexual steroid hormones are studied, only few data are available in feline species, and especially about domestic cats (Terwissen et al., 2014).

Although nails/claws can be supposed to be different among species, they are, indeed, very similar from an anatomic point of view, enclosing in all cases the distal phalanx (Dyce et al., 2013). However, differently by many other species, the claws are retractable in the cat (Homberger et al., 2009), so that they are protected by consumption and could grow more quickly than human nails (Erickson, 2013). On average, the cats' claws have a growth rate of about 2mm/week, and a complete change is estimated to occur in 6-9 months (Erickson, 2013). Another characteristic of cats' claws is the "claw-shedding" (Homberger et al., 2009), by which, periodically, they lose the external cornified claw sheath at the same time maintaining intact the internal layers (De Weerdt, 1927; Homberger et al., 2009).

A picture of the long-term accumulation of sexual steroid hormones, measurable in a single collection of coat and claws, could be a promising tool for the future detection of physiologic conditions such as puberty ascertainment, or for the diagnosis of reproductive disorders, such as estrus or male sexual behavior in gonadectomized female and male cats, and unilateral cryptorchid cats that underwent gonadectomy of only the descended testis. However, before a real and practical application of a new technique, a preliminary study on animals of known reproductive status is needed, in order to define specific reference values for both matrices, for each reproductive status, in both sexes.

Therefore, because of all the reasons reported above, the aims of the present study were to: 1) assess the usefulness of coat and dewclaws for the measurement of long-term accumulation of sexual steroid hormones in domestic cats; 2) assess possible differences in coat and dewclaws concentrations of E2 and T between males and females in 3 different reproductive statuses (pubertal, prepubertal and gonadectomized); 3) verify possible differences of hormones among each reproductive status in males and females.

Materials and methods

Ethics

The study was performed in accordance with the ethical guidelines provided by the animal welfare committee and all the procedures were carried out according to the Italian legislation about animal care (DL 116, 27/01/1992) and to the European Guidelines on Animal Welfare (Directive 2010/63/EU). A written informed consent was signed by the owners, giving the permission to submit each cat to the collection of coat and dewclaws samples, and allowing the record of clinical data for research purposes.

Animals

The study was performed on 55 (27 females and 28 males) domestic cats belonging to private owners. They were of different cat breeds, healthy and in good general conditions.

From each enrolled cat, data about age and reproductive status were recorded and the animals were classified as follows: gonadectomized (only cats gonadectomized by at least 6 months were enrolled); pubertal (cats aged > 6 months, with evidence of puberty such as the occurrence of first heat in females, observed at least two months before entering the study; sexual behavior observed at least two months before entering the study and penile spines presence at the time of study, in males); prepubertal (cats 4 - 5 months old, without any sign of puberty).

Coat and dewclaws collection

During routine clinical examination, the cats were submitted to coat and dewclaws collection.

The coat was collected by shaving an area of about 5 cm^2 from the dorsal surface of the forearm. The shaving was performed by a razor (TN2300 Nomad, Rowenta® spa, Milan, Italy) to allow the

collection of the coat at the level of the skin. The collected coat was immediately put into a uniquely-coded paper envelope and stored at room temperature until analysis.

The tip of the claws belonging to the 1st digit of both forelegs was collected by clipping and, then, the two samples were pooled together (dewclaws sample). Dewclaws were immediately placed in uniquely-coded paper envelopes and stored at room temperature until analysis.

Both coat and dewclaws were collected thanks to a gentle handling of the cats, without a real restraint.

After every coat and dewclaw collection, the razor and the claw clipper were disinfected with a 70% alcohol solution.

Hormone analyses

Coat strands and dewclaws were washed in 3-mL isopropanol to ensure the removal of any steroids on their surface. Coat and dewclaws steroids were extracted with methanol and measured by radioimmunoassay (RIA). The concentrations of 17β-estradiol and testosterone were measured using a solid-phase microtiter RIA. In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Sciences Inc.) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.5) and incubated overnight at 4°C with 200 µL of the antibody serum diluted 1:80,000 for 17β-estradiol and 1:160,000 for testosterone. The cross-reactivities of the anti-17β-estradiol antibody with other steroids were as follows: 17β-estradiol, 100 %; estrone, 2.5 %; estriol, 0.12 %; 17β-estradiol-(B—D-glucuronide), 0.04 %; 17β-estradiol-3-sulfate 0.012 %; DHEA, 0.007 %; 17α-estradiol, < 0.0.4%; progesterone, < 0.0.4 %; testosterone, < 0.0.4 %; androstenedione, < 0.0.4 %; estrone-3-sulfate, < 0.0.4 %. The cross-reactivities of the anti-testosterone antibody with other steroids were as follows: testosterone, 100 %; 5α-dihydrotestosterone, 43.2 %; 5α-androstanedione, 33.1 %; 5β-androstanedione, 11.4 %; 5α-androstan-3α,17β-diol, 9.4 %; androstenedione, 0.4 %; testosterone 17β-glucuronide, 0.09 %; progesterone, DHEA, 17β-estradiol, androsterone-3-glucuronide, 0.01 %; androsterone-3-glucuronide, 0.006 %; cortisol, < 0.001 %. After washing the plate with RIA buffer, the standards (5–200 pg/well), the quality-control extract, the test extracts, and the tracer (17β-estradiol [2,4,6,7-16-17-^{3}H (N)] and testosterone [1,2,6,7-^{3}H (N)]) were added, and the plate was incubated overnight at 4°C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL of scintillation cocktail, the plate was counted on a β-counter (Top-Count; PerkinElmer Life Sciences Inc.). The intra- and inter-assay coefficients of variation were 3.7

and 12.1, 4.40 and 11.5% for 17β-estradiol and testosterone, respectively. The sensitivities of the assays were 0.77 pg/well and 0.33 pg/well for 17β-estradiol and testosterone, respectively.

Statistical analysis

Due to the wide data dispersion and the limited number of subjects in some categories, possible differences in coat and dewclaws concentrations of E2 and T, between males and females in the 3 reproductive statuses (gonadectomized, prepubertal, pubertal), were assessed by Robust ANOVA, followed by post Hoc test. Possible differences of the hormones studied among the three reproductive statuses within each sex, were also assessed with the same statistical test. Statistical significance was set for $p<0.05$ (JASP®, ver 9 for Windows platform).

Results

Classification of the cats in the reproductive statuses and age of the subjects

In the overall 55 cats enrolled in the present study, the age ranged between 4 and 156 months, with an average of 29 ± 35.59 months.

Nine cats were classified in the group of gonadectomized cats, 3 males and 6 females. Their age ranged between 36 and 156 months, with an average age of 81 ± 38.40 months. The interval of time elapsing between gonadectomy and the enrollment in the study was 69 ± 38.40 months, with a range of 24-144 months. In none of them, sexual behavior was observed after gonadectomy.

Thirty-four cats were classified in the pubertal group, 19 males and 15 females. In these cats, age ranged between 7 and 120 months, with an average age of 26 ± 30.31 months.

Twelve cats were classified in the prepubertal group, 3 males and 9 females. Their age ranged between 4 and 5 months, with an average age of 4.5 ± 0.52 months.

Coat and dewclaws collection

Collection of the coat and dewclaws was always easily performed, thanks to a gentle handling, without distress for the animals. The mean ± SD amounts of dewclaws samples were 8.4 ± 2.24 mg (range: 0.8-14.4 mg), and allowed, in all the cases, the analysis of E2 and T. The mean ± SD amount of coat samples was 90.6 ± 24.4 mg (range: 17.3-126.8 mg), and allowed, in all cases, the analysis of E2 and T.

Concentrations of E2 and T in coat and dewclaws

Data about the concentrations of E2 and T in coat and dewclaws of gonadectomized, prepubertal and pubertal male and female cats are reported in table 1-3, respectively. Data are expressed as median (min-max).

Table 1 – Data expressed as median (min-max) about concentrations of E2 and T in coat and dewclaws of gonadectomized male and female cats

	E2 (pg/mg)		**T (pg/mg)**	
	coat	**dewclaws**	**coat**	**dewclaws**
Gonadectomized males (n=6)	1.22 (0.96-1.59)	1.60 (1.35-7.87)	0.99 (0.72-1.45)[a]	1.84 (1.12-3.97)
Gonadectomized females (n=3)	1.11 (1.10-1.12)	0.94 (0.74-1.14)	1.21 (1.14-1.27)[b]	0.45 (0.40-0.49)

[a,b] within columns denotes significant differences ($p<0.01$)

Table 2 – Data expressed as median (min-max) about concentrations of E2 and T in coat and dewclaws of prepubertal male and female cats

	E2 (pg/mg)		**T (pg/mg)**	
	coat	**dewclaws**	**coat**	**dewclaws**
Prepubertal males (n=3)	1 (0.95-1.05)	1.30 (1.12-1.49)	1.19 (1.15-1.22)	1.41 (1.09-1.72)
Prepubertal females (n=9)	1.48 (1.04-3.68)	1.10 (0.77-2.29)	1.12 (0.76-1.37)	0.84 (0.30-1.84)

Table 3 – Data expressed as median (min-max) about concentrations of E2 and T in coat and dewclaws of pubertal male and female cats

	E2 (pg/mg)		**T (pg/mg)**	
	coat	**dewclaws**	**coat**	**dewclaws**
Pubertal males (n=19)	1.18 (0.70-1.59)	1.34 (0.48-3.82)	1.70 (1-3.01)[a]	1.11 (0.48-7.51)
Pubertal females (n=15)	1.13 (1-1.80)	1.34 (0.30-6.38)	1.02 (0.66-3.43)[b]	1.08 (0.40-3.55)

[a,b] within columns denotes significant differences ($p<0.01$)

Table 4 shows the concentrations of E2 and T in coat and dewclaws of female cats, classified according to the reproductive status (gonadectomized, pubertal, prepubertal).

Table 4 - Concentrations of E2 and T in coat and dewclaws of male cats, expressed as median (min-max), classified according to the reproductive status (gonadectomized, pubertal, prepubertal)

Males (n=28)	**E2 (pg/mg)**		**T (pg/mg)**	
	coat	**dewclaws**	**coat**	**dewclaws**
Gonadectomized males (n=6)	1.22 (0.96-1.59)	1.60 (1.35-7.87)	0.99 (0.72-1.45)	1.84 (1.12-3.97)
Prepubertal males (n=3)	1 (0.95-1.05)	1.30 (1.12-1.49)	1.19 (1.15-1.22)[a]	1.41 (1.09-1.72)
Pubertal males (n=19)	1.18 (0.70-1.59)	1.34 (0.48-3.82)	1.70 (1-3.01)[b]	1.11 (0.48-7.51)

[a,b] within columns denotes significant differences ($p<0.05$)

Table 5 shows the concentrations of E2 and T in coat and dewclaws of male cats, classified according to the reproductive status (gonadectomized, pubertal, prepubertal).

Table 5 - Concentrations of E2 and T in coat and dewclaws of female cats, expressed as median (min-max), classified according to the reproductive status (gonadectomized, pubertal, prepubertal)

Females (n=27)	**E2 (pg/mg)**		**T (pg/mg)**	
	coat	**dewclaws**	**coat**	**dewclaws**
Gonadectomized females (n=3)	1.11 (1.10-1.12)	0.94 (0.74-1.14)	1.21 (1.14-1.27)	0.45 (0.40-0.49)
Prepubertal females (n=9)	1.48 (1.04-3.68)	1.10 (0.77-2.29)	1.12 (0.76-1.37)	0.84 (0.30-1.84)
Pubertal females (n=15)	1.13 (1-1.80)	1.34 (0.30-6.38)	1.02 (0.66-3.43)	1.08 (0.40-3.55)

Discussion

The results of the present study must be considered only preliminary from several aspects: because of the limitations about these new matrices tested in the cat; limited number of total animals enrolled and subdivision in the different reproductive statuses within each sex; limitations of studied hormones; limitations of reproductive status assessment and enrollment of animals.

The usefulness of hair/coat and nails/claws as new matrices for long-term investigations about hormonal changes was proved in humans and animals, as reported above. In animals, the use of these matrices was advantageous because it fits well with the need of respecting animal welfare, with lack of invasiveness at collection and with the reduction of the number of samplings, particularly important in the cat. Some advantages and limitations have been detected about the hair as a matrix for hormonal investigations in humans and animals, while little information is available about the nails: about this matrix, some published data are reported in humans (Tegethoff et al., 2011) and dogs (Veronesi et al., 2015). Moreover, in the cat, the claws have some peculiarities that make this matrix possibly different from other species: retractability, scratching and claw-shedding, as reported above. For these reasons, in the present study only the dewclaws, less submitted to scratching, were collected. About the hair/claws samples, only few data are available about the possible influence of some factors in the accumulation of hormones. Notably, region of collection and hair (coat) color are among the parameters analyzed for a possible influence on the accumulation of hormones. However, up to now those results are conflicting and available only in dogs, not in the cat. However, in the present study, coat was collected in all cases from the dorsal surface of the forearm and coat color was not taken into consideration, because of the high heterogeneity of coat colors. However, for a better understanding of the real usefulness of these matrices for the investigation of long-term changes of sex steroids in the cat, these aspects deserve to be investigated. In both coat and claws, the possible role played by pigmentation (and color) in the process of hormones accumulation should be clarified, as well as the possible role played by the different length of the hair shaft. Furthermore, specifically for the claws, it will be necessary to assess possible similarities or differences in hormones accumulation between the dewclaws and the claws belonging to the other digits. About the cats' coat, it remains to be clarified the possible effect played by the body region of collection, by the color, and possibly by the different lengths on accumulation of hormones. Other than this, it is desirable to consider the possible differences related to the breed.

Nonetheless, the preliminary results obtained in this study should be cautiously considered, due to the small number of enrolled animals (especially for some reproductive statuses in both sexes). In fact, only 3 prepubertal males and 3 gonadectomized females were enrolled. Moreover, also the enrollment of prepubertal subjects could have been designed upon more strict rules. In fact, due to the gradual process of puberty attainment, it is not possible to state that all the cats classified as prepubertal were actually far from the onset of puberty at the time of sampling, especially the males. Another limitation of the present study concerns the pubertal females. Although the measurement of 17β-estradiol concentrations in coat and dewclaws depict a retrospective long-term accumulation, indeed, the hormonal concentrations measured could have been influenced by the number of estrus phases repeated before sampling and, in turn, by the time elapsed from the starting of the breeding season and the collection of hair and dewclaws. Therefore, further investigations should be designed with more accurate selection criteria.

About the hormones measured, although T and E2 represents the major sex steroids for males and females, respectively, it could be interesting to include some other hormones such as dehydroepiandrosterone sulfate, progesterone and cortisol. The first as precursor for sex steroids production, the second to detect possible spontaneous ovulatory cats, the last as recognized marker of stress that could be associated to reproduction and, in particular, to puberty.

A more complete hormonal investigation, coupled to a more precise animal selection, will provide basic data for the further application of these matrices as a tool for investigating both some physiologic features of feline reproduction and reproductive disorders.

Besides the limitations, several advantages evidenced by the present study deserve to be mentioned. The collection of the tip of the dewclaws by clipping was easily performed in all the animals thanks to a gentle handling, never required a real restraint, and never caused restlessness. The coat was also collected without disturbing the animals, thanks to the use of a low-noise razor. This aspect is very important from an animal welfare perspective and especially when concerning cats. In addition, it was also accepted without reluctance by all the owners. Storage of samples was also very interesting. In fact, both hair and nails were stored at room temperature in uniquely-coded paper envelopes, without time limits between collection and storage, and without the need for having storage instruments. Lastly, the amount of sample needed for the analyses of both T and E2 was limited: 90.6 ± 24.4 mg (range: 17.3 - 126.8 mg) for the coat, and 8.4 ± 4.24 mg (range: 0.8 - 24.4 mg) for the dewclaws, evidencing, once more, the usefulness of the coat and the claws for long-term, retrospective measurement of hormones

accumulation also in the cats, as previously demonstrated in other species and in cats only for coat (Terwissen et al., 2014).

The statistical analysis on these preliminary data showed that only T concentration in coat was different between male and female pubertal ($p<0.01$) and between male and female gonadectomized ($p<0.01$) cats. In detail, T concentrations in coat was, as expected, higher in male than female pubertal cats, while T concentrations in coat were higher in female than male gonadectomized cats. As a speculation, this last finding could be related to an increase of androgens before their conversion to estrogens, and the higher concentrations found in females than males could reflect a more precocious development in females than males.

Within each sex, T concentrations in coat were, as expected, significantly ($p<0.05$) higher in pubertal than prepubertal male cats, while no differences were found within the females, for both hormones. Regarding the concentration of T in the coat, in a study on domestic cats, there was no statistical difference between intact and castrated males (Terwissen et al., 2014). Even when the concentrations of E2 in coat were analyzed in females, no statistical differences among the different reproductive statuses (intact, spayed, estrus, pregnant) were found (Terwissen et al., 2014). However, in that study the hormonal analyses were performed by Enzyme Immuno-Assay (EIA) technique, so that the results obtained are not directly comparable with the findings of the present study. Even in bears, the assessment of E2 measured in hair did not allow a differentiation between sex and reproductive statuses in the different times of the year, given the seasonality of reproduction in this species (Cattet et al., 2017). Also in this case, however, a direct comparison among the values is not possible given the different method of analysis used.

The complete absence of significant differences in hormones measured in the dewclaws seems to suggest that, relying on these preliminary data, coat is more suitable than dewclaws for the study of possible retrospective, long-term accumulation of E2 and T in domestic cats. This finding needs further investigations, but could be related to the specific nature of the claws in cats, in which the typical phenomena of scratching and claw-shedding could have impaired the correct measurement of these hormones.

Conclusions

In conclusion, the preliminary results of the present study proved that E2 and T concentrations are measurable in the coat and in the dewclaws of domestic cats. The coat seems to be more useful than dewclaws as matrix for the measurement of long-term concentration of T and E2 in the domestic cat. In fact, when used for the definition of the reproductive status within each sex, only T concentrations in coat showed significant differences between pubertal males and females and between gonadectomized male and females. Only concentrations of T in the coat were different between pubertal and prepubertal male cats. These results provide further knowledge about the use of these matrices in this species, however future investigations should be designed to deepen the knowledge about the use of these matrices, collected only once, to distinguish prepubertal, pubertal and gonadectomized subjects within a sex in the domestic cat. Therefore, the study needs to be implemented, enrolling a more suitable number of cats, better distributed within each sex and within each reproductive status. Apart of the total number of animals, also the requisites for the enrollment and the definition of reproductive statuses should be better defined, and the timing of coat and dewclaws collections should be better scheduled within the breeding season in pubertal cats. At last, some intrinsic aspects specifically related to coat and claws matrices should be better investigated for a more suitable use of these interesting matrices in the study of long-term hormonal changes related to reproduction in domestic cats.

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MEASUREMENT OF 17β-ESTRADIOL, TESTOSTERONE, CORTISOL AND DEHYDROEPIANDROSTERONE-SULFATE CONCENTRATIONS IN ONE-SHOT SAMPLE OF COAT AND DEFINITION OF THE REPRODUCTIVE STATUS IN MALE AND FEMALE DOGS: PRELIMINARY RESULTS

Introduction

In mammals, the beginning of reproduction is marked by the onset of puberty, attained thanks a complex, gradual interplay of endocrinological and metabolic factors (Kinder et al., 1995; Hiney et al., 1996; Gobello, 2014), including a complex interaction among the external environment, the nervous and endocrine systems. The interactions between the Hypothalamic-Pituitary-Adrenal (HPA) and Hypothalamic-Pituitary-Gonadal (HPG) axes lead to physical, behavioral and hormonal changes that, in turn, causes the beginning of reproduction. In the male, puberty attainment is detected by the beginning of spermatogenesis and first ejaculation (Johnston et al., 2001a; Sjaastad et al., 2013), or even by the observation of sexual behavioral traits (Gobello, 2014). In the female, puberty is detectable by the onset of the first heat, due to the beginning of the ovarian activity (Sjaastad et al., 2013), responsible for production of gametes and ovarian hormones. In the dog (*Canis lupus familiaris*), the proestrus is detectable by the appearance of the typical vaginal bleeding, and could be considered as a sign of puberty attainment (Johnston et al., 2001b). In dogs, puberty attainment is influenced by many factors, in which body-size plays a central role. Generally, small- and miniature-sized dogs reach puberty at the age of 5-6 months, while large- and giant-sized dogs attain puberty only after 18 months of age (Gobello, 2014).

As a result of the stimulation of the HPG axis, puberty is characterized by the increase of circulating sexual hormones, testosterone (T) in males, estrogens (with 17β-estradiol, known as E2, as the main representative) and progesterone in females (Alotaibi, 2019). However, some other hormones, such as cortisol (C) and dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEA-S), interplay with sex steroids in the complex mechanisms of puberty attainment. The hormonal patterns occurring during the postnatal period were studied in some domestic species, but few data are available about dogs (Gobello, 2014).

Gonadectomy implies the fall of circulating sexual steroids by the removal of the gonads (ovaries and testes) (Kim et al., 2005), and it is often performed in companion animals to control animal population,

but also for the animal health benefit (Root Kustritz, 2012). Both the concentrations of circulating T and E2 in males and females, respectively, were reported to decrease significantly from one week after gonadectomy (Ibrahim and Zaid, 2017). The basal concentrations of plasma T were reported to be useful to verify the neuter status in a male dog (Ibrahim and Zaid, 2017), while in female bitches the measurement of plasma E2 two hours after an injection of GnRH could be used to discern between functional and non-functional ovarian tissue (De-Gier et al., 2012).

Practical issues related to puberty attainment in dogs should not be underestimated. First, pubertal attainment does not imply the subject is fertile and able to display the typical sexual behavior, so that pubertal dog with suspected infertility or sexually immature, were reported (Corrada et al., 2006). Second, the breeders could sometimes face economic losses due to retardation in puberty attainment.

Thus, increasing the knowledge about this phase, and especially a more specific deepening of the interplays occurring during this phase between HPA and HPG axes, is strongly recommended (Goel et al., 2014) in all species. Improvement of knowledge about hormonal variations around puberty in companion animal were reported to be desirable (Gobello, 2014), as well as the possible interaction between the HPA and the HPG axes. However, also the availability of new, noninvasive tools for the recognition of gonadectomized companion animals are requested. In fact, sometimes, neutered/spayed dogs can display sexual behavior that could be related to behavioral disturbances or to a real hormonal stimulation, mainly due to troubles related to the gonadectomy. In males, this condition can also be related to unilateral orchidectomy in cryptorchid subjects. Usually, these conditions are investigated by the noninvasive ultrasound examination, but often require the additional blood hormonal analysis and exploratory laparotomy, to provide a final diagnosis.

Therefore, the availability of new matrices, collectable without invasiveness, could represent an interesting new tool for both the detection of puberty attainment and the definition of the actual gonadectomized status.

When hormonal analysis is concerned, the measurement can be performed on several biological matrices, such as blood, saliva, urines, feces, and in some cases milk (Mesarčová et al., 2017). Each one of these matrices has own advantages and disadvantages, so that their choice should rely on specific characteristics. One of the issues to consider is the invasiveness of collection, in a perspective of animal welfare. From the same standpoint, blood should not be considered as a first-line choice. Saliva is collectable with minor disturbance for the animals, and urines and feces are collectable without interference with animal welfare, although sometimes it could be difficult to practice (Schell et al., 2017). The second issue is based on the time-window in which hormonal changes are investigated.

In fact, blood (or plasma or serum) reports concentrations detectable in the time in which the collection was performed, while urines depict hormonal concentrations of a single time point or of the last 12-24 hours (Bergamin et al., 2019). Therefore, these matrices are not useful for studies investigating long-term hormonal changes, because they require the repetition of a certain number of samplings. A last consideration concerns the method of sample storage, in all these cases represented mostly by freezing. Since it overcomes these necessities, hair (or coat) proved to be a useful matrix for studies about long-term hormonal changes (Bergamin et al., 2019). The hair/coat is, indeed, collectable without invasiveness (Accorsi et al., 2008), provide retrospective information about long-term hormonal changes (Accorsi et al., 2008; Veronesi et al., 2015; Mesarčová et al., 2017) and can be stored at room temperature (Veronesi et al., 2015; Voegel et al., 2018). The hair/coat proved to be useful for the study of chronic stress, measuring the concentrations of C accumulated in a long time (Voegel et al., 2018) in both humans and many animal species, including the dog. Cortisol is considered the endpoint of the mechanism of stress and, therefore, useful as a marker of stress, even if also the measurement of DHEA and DHEA-S was reported to be useful for assessing stress and HPA activation (Bergamin et al., 2019). About DHEA and DHEA-S analysis, when the technique used is unable to discern between the measurement of DHEA and DHEA-S, the results reported could be reported as the sum of the two compounds and indicated as DHEA(S) (Whitham et al., 2020). However, a study published in 1998 by Yang and colleagues reported the possibility to use this matrix also for the detection of sexual steroid hormones. Therefore, recently, some studies reported the measurement of sexual hormones in the hair of humans (Grotzinger et al., 2018; Jahangard et al., 2019) and animals (Devi et al., 2018; Tallo-Parra et al., 2018; Bergamin et al., 2019; Ventrella et al., 2020). Sexual steroids were also reported to be involved in stress response (Bergamin et al., 2019), evidencing the close interaction between the HPA and HPG axes.

Many factors were reported to influence the measurement of some hormones in the hair (Heimbürge et al., 2019), even if, sometimes, conflicting results are provided. For instance, although the region of the body from which the hair/coat sampling is performed was supposed to influence the C concentrations, Schell et al. (2017), evidenced that, in coyotes (*Canis latrans*), the concentrations of C and T did not differ in hair/coat samples collected by diverse regions of the body. At last, also the hair/coat follicle was recognized as a local source for C production, so that its measurement could not be addressed only to the central HPA axis activation (Bennett and Hayssen, 2010). Moreover, also the color of the hair/coat were suggested to influence some hormonal accumulations in the hair/coat, but conflicting results are at present available, as reported above (Heimburge et al., 2019).

The property of the hair/coat to provide a retrospective picture of long-term hormone accumulation seems to suggest that even only a single hair/coat collection could be reliable for the assessment of a cumulative hormonal stimulation within an individual. According to this hypothesis, the long-term hormonal accumulation in the hair/coat collected only once could be a promising tool for detecting puberty ascertainment or reproductive disorders, such as sexual behavior in gonadectomized female and male dogs, and unilateral cryptorchid dogs that were submitted to gonadectomy. However, before a real practical application of a new technique, a preliminary study on animals of known reproductive status is needed, in order to define the reference values related to the matrix for each reproductive status in both sexes.

The aims of the present study were therefore to: 1) assess the reliability of a single coat collection to discern between males and females belonging to three known different reproductive status (prepubertal, pubertal, and gonadectomized); 2) verify possible differences of hormones among each reproductive status in males and females. To better understand the possible interaction between the HPA and HPG axes, other than E2 and T, known as the main sexual steroids, also C and DHEA-S were investigated.

Materials and methods

Ethics

The study was performed in accordance with the ethical guidelines provided by the animal welfare committee and all the procedures were carried out according to the Italian legislation about animal care (DL 116, 27/01/1992) and to the European Guidelines on Animal Welfare (Directive 2010/63/EU). A written informed consent was signed by the owners, giving the permission to submit each dog to the collection of coat samples, and allowing the record of clinical data for research purposes.

Animals

The study was conducted on 40 dogs of private owners, belonging to many different breeds, 20 males and 20 females. All the dogs were healthy, in good general conditions, and with BCS of 2.5-3 on a scale of 1 to 5, admitted for routine clinical examinations.

According to signalment data, dogs were classified as follows: gonadectomized (only dogs gonadectomized by at least 6 months were enrolled); pubertal (dogs aged > 6 months, with evidence of puberty such as the occurrence of first heat in females, observed at least two months before entering the

study, and leg lifting during urination in males, observed from at least two months before entering the study); prepubertal (dogs 3-5 months old, without any sign of puberty).

Coat collection

During the routine clinical examination, the dogs were submitted to a single coat collection.

The coat was collected by shaving an area of about 5 cm^2 from the dorsal surface of the forearm in all subjects. Shaving was performed by a razor (TN2300 Nomad, Rowenta® spa, Milan, Italy) to allow the collection of the coat at the level of the skin. The collected coat was immediately put in a uniquely-coded paper envelope, and stored at room temperature until analysis. After every hair collection, the razor was disinfected with a 70% alcohol solution.

Hormone analyses

Coat strands were washed in 3-mL isopropanol to ensure the removal of any steroids on their surface. Coat steroids were extracted with methanol and measured by radioimmunoassay (RIA). The concentrations of cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEA-S), 17β-estradiol and testosterone were measured using a solid-phase microtiter RIA. In brief, a 96-well microtiter plate (OptiPlate; PerkinElmer Life Sciences Inc.) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.5) and incubated overnight at 4°C with 200 µL of the antibody serum diluted 1:20,000 for cortisol, 1:2,000 for DHEA, 1:800 for DHEA-S, 1:80,000 for 17β-estradiol and 1:160,000 for testosterone. The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisol 100%, cortisone 4.3%, corticosterone 2.8%, 11-deoxycorticosterone 0.7%, 17-hydroxyprogesterone 0.6%, dexamethasone 0.1%, progesterone, 17-hydroxypregnenolone, DHEA-S, androsterone sulphate and pregnenolone < 0.01%. The cross-reactivities of the anti-DHEA antibody with other steroids were as follows: DHEA, 100%; pregnenolone, 0.1; dihydrotestosterone, 0.05; DHEA-S, 0.02; testosterone, <0.01%; androsterone, <0.01%; epiandrosterone, <0.01%; estradiol, <0.01%; progesterone, <0.01%; cholesterol, <0.01%; estrone, <0.01%. The cross-reactivities of the anti-DHEA-S antibody with other steroids were as follows: DHEA-S, 100%; androstenedione, 0.2%; DHEA, <0.01%; androsterone, <0.01%; testosterone, <0.01%. The cross-reactivities of the anti-17β-estradiol antibody with other steroids were as follows: 17β-estradiol, 100 %; estrone, 2.5 %; estriol, 0.12 %; 17β-estradiol-(B—D-glucuronide), 0.04 %; 17β-estradiol-3-sulfate 0.012 %; DHEA, 0.007 %; 17α-estradiol, < 0.0.4%; progesterone, < 0.0.4 %;

testosterone, < 0.0.4 %; androstenedione, < 0.0.4 %; estrone-3-sulfate, < 0.0.4 %. The cross-reactivities of the anti-testosterone antibody with other steroids were as follows: testosterone, 100 %; 5α-dihydrotestosterone, 43.2 %; 5α-androstanedione, 33.1 %; 5β-androstanedione, 11.4 %; 5α-androstan-3α,17β-diol, 9.4 %; androstenedione, 0.4 %; testosterone 17β-glucuronide, 0.09 %; progesterone, DHEA, 17β-estradiol, androsterone-3-glucuronide, 0.01 %; androsterone-3-glucuronide, 0.006 %; cortisol, < 0.001 %. After washing the plate with RIA buffer, the standards (5–200 pg/well), the quality-control extract, the test extracts, and the tracer (hydrocortisone {cortisol [1,2,6,7-^{3}H (N)]-}, DHEA [1,2,6,7-^{3}H (N)], DHEA-S [1,2,6,7-^{3}H (N)], 17β-estradiol [2,4,6,7-16-17-^{3}H (N)] and testosterone [1,2,6,7-^{3}H (N)]) were added, and the plate was incubated overnight at 4°C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL of scintillation cocktail, the plate was counted on a β-counter (Top-Count; PerkinElmer Life Sciences Inc.). The intra- and inter-assay coefficients of variation were 3.7 and 10.1%, 3.8 and 10.6%, 3.2 and 11.8, 3.7 and 12.1, 4.40 and 11.5% for cortisol, DHEA, DHEA-S, 17β-estradiol and testosterone, respectively. The sensitivities of the assays were 1.23 pg/well, 0.62 pg/well, 0.54 pg/well, 0.77 pg/well and 0.33 pg/well for cortisol, DHEA, DHEA-S, 17β-estradiol and testosterone, respectively.

Statistical analysis

Due to the wide data dispersion and the limited number of subjects in some categories, possible differences of E2, T, C and DHEA(S) concentrations in coat between males and females in the 3 reproductive statuses were assessed by Robust ANOVA, followed by post Hoc test. Possible differences of the hormones studied among the three reproductive statuses within each sex, were also assessed with the same statistical test. Statistical significance was set for $p<0.05$ (JASP®, ver 9 for Windows platform).

Results

According to the classification of the reproductive status of each dog, 5 males and 5 females were prepubertal (mean age: 4.4 ± 0.40 months); 5 males and 5 females were gonadectomized (mean age: 29.7 ± 23.3 months); 10 males and 10 females were pubertal (mean age: 24 ± 15.78 months).

Coat collection was easily performed in all the subjects, without restraint, and provided an amount of 101.1 ± 20.7 mg (mean ± SD), with a range of 9.7 - 118.4 mg of coat sample allowing, in all the subjects, the analysis of all the scheduled hormones.

The statistical analysis showed the non-normal distribution of data, so that results about hormonal concentrations in coat were expressed as median (min-max) values.

Table 1-3 showed, respectively, E2, T, C and DHEA(S) concentrations in coat, expressed as median (min-max) in male and female dogs, in the 3 reproductive statuses (gonadectomized, pubertal, prepubertal).

Table 1 – Data expressed as median (min-max) about E2, T, C and DHEA(S) concentrations in coat of gonadectomized male and female dogs

	E2 (pg/mg)	**T (pg/mg)**	**C (pg/mg)**	**DHEA(S) (pg/mg)**
Gonadectomized males (n=5)	1.30 (1.10-3.40)	2.50 (1.20-3.10)	2.90 (2.10-3.50)	10.70 (5.40-12.30)
Gonadectomized females (n=5)	2.30 (2.10-2.70)	1.50 (1.30-1.90)	3.10 (2.60-3.80)	8.70 (7.50-10.00)

Table 2 - Data expressed as median (min-max) about E2, T, C and DHEA(S) concentrations in coat of prepubertal male and female dogs

	E2 (pg/mg)	**T (pg/mg)**	**C (pg/mg)**	**DHEA(S) (pg/mg)**
Prepubertal males (n=5)	1.70 (1.10-2.10)	1.70 (1.50-1.80)[a]	2.80 (2.60-2.80)	10.10 (6.20-22.30)
Prepubertal females (n=5)	2.30 (1.80-2.50)	1.00 (0.80-1.50)[b]	2.20 (1.50-2.90)	8.50 (7.30-10.40)

[a,b] within columns denotes significant differences ($p<0.01$)

Table 3 - Data expressed as median (min-max) about E2, T, C and DHEA(S) concentrations in coat of pubertal male and female dogs

	E2 (pg/mg)	T (pg/mg)	C (pg/mg)	DHEA(S) (pg/mg)
Pubertal males (n=10)	2.15 (1.70-3.20)	2.60 (2.20-4.10)[a]	4.20 (3.20-10.10)[a]	15.70 (4.40-58.20)[c]
Pubertal females (n=10)	2.20 (1.50-2.90)	1.20 (0.60-2.90)[b]	2.90 (1.90-3.50)[b]	2.90 (1.90-11.40)[d]

[a,b] within columns denotes significant differences ($p<0.01$); [c,d] within columns denotes significant differences ($p<0.01$)

Table 4 showed E2, T, C and DHEA(S) concentrations in coat, expressed as median (min-max), in female dogs, classified according to the reproductive status (gonadectomized, pubertal, prepubertal). Table 5 showed E2, T, C and DHEA(S) concentrations in coat, expressed as median (min-max), in male dogs, classified according to the reproductive status (gonadectomized, pubertal, prepubertal).

Table 4 - E2, T, C and DHEA(S) coat concentrations, expressed as median (min-max) in female dogs, classified according to the reproductive status (gonadectomized, pubertal, prepubertal)

Female dogs (n=20)	E2 (pg/mg)	T (pg/mg)	C (pg/mg)	DHEA(S) (pg/mg)
Gonadectomized (n=5)	2.30 (2.10-2.70)	1.50 (1.30-1.90)	3.10 (2.60-3.80)	8.70 (7.50-10.00)[a]
Pubertal (n=10)	2.20 (1.50-2.90)	1.20 (0.60-2.90)	2.90 (1.90-3.50)	2.90 (1.90-11.40)[b]
Prepubertal (n=5)	2.30 (1.80-2.50)	1.00 (0.80-1.50)	2.20 (1.50-2.90)	8.50 (7.30-10.40)[c]

[a,b] within columns denotes significant differences ($p<0.01$); [b,c] within columns denotes significant differences ($p<0.05$)

Table 5 - E2, T, C and DHEA(S) coat concentrations, expressed as median (min-max) in male dogs, classified according to the reproductive status (gonadectomized, pubertal, prepubertal)

Male dogs (n=20)	E2 (pg/mg)	T (pg/mg)	C (pg/mg)	DHEA(S) (pg/mg)
Gonadectomized (n=5)	1.30 (1.10-3.40)	2.50 (1.20-3.10)	2.90 (2.10-3.50)[e]	10.70 (5.40-12.30)
Pubertal (n=10)	2.15 (1.70-3.20)	2.60 (2.20-4.10)[a]	4.20 (3.20-10.10)[c]	15.70 (4.40-58.20)
Prepubertal (n=5)	1.70 (1.10-2.10)	1.70 (1.50-1.80)[b]	2.80 (2.60-2.80)[d]	10.10 (6.20-22.30)

[a,b] within columns denotes significant differences ($p<0.05$); [c,d] within columns denotes significant differences ($p<0.001$); [c,e] within columns denotes significant differences ($p<0.001$)

Discussion

Data from the present study confirm, once more, the usefulness of dog's coat as a matrix for the retrospective measurement of hormones accumulated in a long-term manner. Moreover, the results of the present study evidence that even a single coat collection allows the retrospective measurement of several hormones previously accumulated in the hair shaft.

The collection of the coat from the dorsal surface of the forearm proved to be easy, quick and did not cause any disturbance to all the enrolled animals, providing further evidence that this matrix can be collected without invasiveness, disturbance, pain and fear for the dogs.

In the present study, dogs belonging to multiple breeds were enrolled, and the length and the color of the coat samples collected were different. However, because of the high heterogeneity of the relatively small number of subjects enrolled, the possible effects played by breed, by coat length and color on E2, T, C and DHEA(S) long-term accumulation in coat was not evaluated. It will be interesting, therefore, to assess the possible effects of these factors on accumulation of hormones in the coat, although available data from humans and animals have provided conflicting results, as reported above.

When data about long-term and retrospective accumulation of hormones were provided, a first statistical analysis was performed to assess possible differences in the accumulation of hormones in coats of males and females for each considered reproductive status. In prepubertal dogs, T concentrations in coat were significantly higher in males than females ($p<0.01$).

In pubertal dogs, when compared to females, male dogs showed higher T ($p<0.01$), DHEA(S) ($p<0.001$) and C ($p<0.01$) concentrations in hair. The findings of higher T and DHEA(S) concentrations in hair of pubertal males are not surprising, due to the recognized androgenic role of testosterone and of DHEA as androgen precursor (Leowattana, 2004; Whitham et al., 2020). Higher concentrations of C in hair of males than females seem to suggest that the pubertal state could be more stressful for males. This finding opens a multitude of interesting questions about the association between sexual activity, behavior and stress, topics that do not fit with the aims of the present study and that need more focused studies (Grotzinger et al., 2018). In gonadectomized dogs, no differences between female and male dogs were found. This result could be explained by the reduction of circulating sexual steroids in both sexes, but also by a similar allostasis after gonadectomy in both male and female dogs. In a work on Poodle dogs, concentrations of T in hair of intact males were higher than in intact females (Calamari et al., 2020). However, when concentrations of T were measured from hair of castrated males, the concentrations were not significantly different from intact females (Calamari et al., 2020). Although these results are very interesting, caution should be taken when comparing the results of the present study with the study of Calamari and coworkers (2020), as in their work the division of the subjects per category was not established with the same criteria of the present thesis and the age of the subjects enrolled was not specified. Other than this, as recently recommended, when subjects with a reproductive status of neutered or spayed are included in a study, it should always be specified from how long the subject underwent gonadectomy (Schrank and Romagnoli, 2020). This is even more important given the peculiarity of long and retrospective time of accumulation of the hair matrix.

On the other hand, however, in brown bears the concentrations of T in hair tended to be higher in females than in males (Cattet et al., 2017). Albeit this comparison is established considering the same matrix and hormone, there are some limits to consider. Firstly, differently to the dog, in bears reproduction is influenced by seasonality. Secondly, the reproductive status in the study of Cattet and colleagues (2017) was set up in a different manner than the present study.

The assessment of possible differences about the long-term concentrations of hormones in coat among the three reproductive statuses within each sex, showed a significantly lower DHEA(S) concentration in pubertal than prepubertal ($p<0.05$) and gonadectomized ($p<0.01$) female dogs. In males, T concentrations in coat were significantly higher ($p<0.05$) in pubertal than prepubertal dogs; C concentrations in coat were also significantly higher in pubertal than prepubertal ($p<0.001$) and gonadectomized ($p<0.05$) dogs. These data seem to suggest that the pubertal status in male dogs entails not only the expectable higher exposition to T in comparison to the prepubertal stage, but also that

pubertal status leads to a higher activation of the HPA axis and, in turn, C secretion that accumulates in the coat. The higher concentrations of C in coat of pubertal than gonadectomized dogs, moreover, seems to suggest that neutering could be associated with a lower activity of the HPA axis. It is therefore possible to speculate that, in male dogs, pubertal status could be considered more stressful than prepubertal or gonadectomized status, but more research is needed to deepen this aspect. As stated above, the sexual activity and behavior could be supposed as a source of stress for the male, needing further focused investigations that are beyond the purpose of the present study. In females, only DHEA(S) was found to be lower in pubertal than prepubertal and gonadectomized bitches. This finding could be supposed to be related to the role of DHEA as hormone, and especially as androgen precursor, arising in males at puberty. However, even if only marginal, a role of DHEA(S) as a precursor for estrogenic production is reported (Lowattana, 2004).

When the hormonal concentrations in coat are considered, it is not possible to compare the data obtained in the present work with results reported in literature about hormones measured in blood or other "instantaneous" matrices. Corroborating this, in dogs, T concentrations in blood were reported to vary coherently with the neutered/spayed status, but not when hair were analyzed (Calamari et al., 2020). Other than that, when T concentrations were considered, the same study did not find any correlation between blood and hair concentrations (Calamari et al., 2020).

In the present study, pubertal males showed a concentration of T in hair with a median of 2.60 pg/mg. This value is lower than the 7.26 ± 1.72 ng/g (mean ± SD) reported for intact males in the work from Calamari and coworkers (2020), but in that study the age of dogs was not specified, and it could be possible that the different time of T accumulation may have played a role in the different concentrations measured. This hypothesis could be supported by the smaller differences observed between the gonadectomized males enrolled in the present study and the castrated males of the study from Calamari and coworkers (2020), in both cases investigating T concentrations in hair/coat. Indeed, in the present study the concentrations of T in coat of castrated males were 2.50 pg/mg (expressed as a median), whilst Calamari and coworkers (2020) reported 3.26 ± 0.94 ng/g (mean ± SD). A real comparison cannot be performed as the study of Calamari and coworkers (2020) did not report the time elapsed from gonadectomy and hair collection, and, nonetheless, also the distinction regarding the breed of the subjects enrolled should be considered. In the present work, the dogs enrolled were from different breeds, whilst Calamari and coworkers (2020) enrolled only Poodle dogs, so that also this factor could have contributed to the different concentrations of hormones observed in these two studies.

A final consideration must be related to the limitations of the present study. Firstly, the collection of a single coat sample, without information about the interval of time between the first appearance and the collection of that sample of coat prevent to understand to which time-window the measured hormones refers. In the present study, therefore, the hormones measured in prepubertal subjects could reflect a continuous accumulation. In pubertal dogs, although at least two months were elapsed between the observation of puberty and the collection of coats, it is possible that the hormones incorporated in the coat following puberty attainment were already measurable in the sample, thus confounding the result obtained. In pubertal bitches, moreover, it is possible to speculate that the estrogenic stimulation of about 10-15 days was not enough to allow a significant (and therefore retrospectively detectable) E2 incorporation in the hair shaft, or it was "diluted" along the time elapsing between estrus and coat collection. Due to the long diestrus phase typical of the dog, it could be interesting to assess if this hypothesis could be applicable also for progesterone concentrations, not analyzed because of the Covid-19 restrictions, as mentioned above.

Conclusions

In conclusion, the results of the present study showed that a single collection of coat in dogs is suitable for the retrospective analysis of E2, T, C and DHEA(S), and that some interesting differences between the two sexes in different reproductive statuses, as well as differences among reproductive statuses within each sex, were found. However, these results must be cautiously considered only preliminary, and this topic needs further in-depth studies. At last, the single collection of coat provides information affected by several factors that need to be better investigated.

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GENERAL DISCUSSION

The results obtained in the three project lines, and reported in the present thesis, demonstrated the usefulness of some matrices collectable without invasiveness for the study of long-lasting phases in companion animal reproduction. In fact, in the studies about pregnancy and post-partum, perinatology, and puberty, coat, claws and fetal fluids proved to be reliable matrices for the retrospective analysis of some long-term hormonal changes. However, fetal fluids are, at present, collectable only once in companion animals, while coat and claws proved to be reliable for longitudinal studies, in which repeated samplings are needed. Given the different peculiarities of coat and claws as compared to fetal fluids, the main characteristics of the first two matrices will be discussed separately from the third one.

As stated above, one of the most important features of coat and claws is to provide a retrospective information about the previous long-term accumulation of hormones. On one hand this characteristic represents a strength, limiting the number of samplings in longitudinal studies. On the other hand, this aspect precludes their use in studies about acute hormonal changes, for which other matrices should be preferred.

Another important characteristic of these matrices is the lack of invasiveness at collection. Indeed, as explained above, the collection of these samples does not need restraint of the animals: most of the time, the animals were already familiar with both coat shaving and claws clipping, this last one routinely performed by the breeders or the owners. The only exception was the collection of claws from puppies enrolled in the study of perinatology. In this case, in fact, puppies were trained to claws clipping during the study. However, in all cases, claws clipping was feasible thanks to a gentle handling of the puppy by the breeder, always for less than 3 minutes, and thanks to clipping only the avascular tip of the claws, avoiding any source of pain, disturbance, fear or distress. Apart of the first collection at birth, immediately after the subsequent collections a positive reinforcement was provide to the puppies in order to accustom them to the procedure.

About the study of puberty, when research was directed to assess the reliability of coat/dewclaws collected only once for measuring hormones in relation to the reproductive status of males and females, young/adult dogs and cats were enrolled. Therefore, some of them were already used to the procedures of coat shaving and claws clipping, while others, especially the youngest, were not. However, also in this case, in all instances collection was easily done, without restraint, but gentle handling was sufficient to prevent disturbance, fear or stress. In this case, also, the total time for the collection never exceeded 3 minutes.

Generally speaking, the property of being collectable without invasiveness is even more important when long-lasting studies are planned and performed, thanks to the reduction of disturbance for the subjects enrolled. This aspect has a double advantage, particularly important for studies on animals: on one hand the undoubted respect of animal welfare from an ethic perspective; on the other hand, the availability of more accurate data, not influenced by the stress of the subjects related to the sampling procedure. The concept of noninvasiveness, moreover, is an essential prerequisite when studies about perinatology or pregnancy and post-partum are concerned. Indeed, any type of disturbance must be avoided during these phases, because distress could bring to detrimental consequences. In the present study, in all cases, samples were collected without annoyance to pregnant and post-partum bitches and to puppies, demonstrating the usefulness of these matrices for studying these delicate physiologic phases. However, it should be noted that also the retrospective property of these matrices, and in turn the reduction of the number of samplings, positively concurred to the advantage of lack of invasiveness. In the present PhD project lines, for all the long-lasting studies the sampling were designed to be performed every 30 days. This interval demonstrated to be feasible to provide suitable amounts of samples, in both coat and claws, to allow the hormonal analyses. It was also suitable to provide reliable hormonal changes information, other than limiting the number of samplings. Moreover, the suitability of a single coat or claws collection for the measurement of hormones in relation to different reproductive statuses in female and male cats and dogs, was assessed. In that case, given the single collection, the possible disturbance to the animals was further restricted, and some interesting information were obtained. However, the measurement of hormones on a single coat or claws collection suffered of a main limitation due to the unknown starting point of hormones accumulation, that could have led to possible misinterpretation of the hormones analyzed.

In the present PhD thesis, other limitations about the use of coat and claws as matrices for the measurement of long-term accumulation of hormones were detected, and need to be further investigated. The first issue concerns the impossibility to compare the results obtained with studies reporting hormonal changes measured in other matrices, given the intrinsic different nature of the diverse matrices, in some cases not retrospective (like blood) or with limited time of retrospectivity (like urines and feces). Therefore, the results obtained in the present research project lines must be considered as a promising starting point for future and more focused studies, aimed to provide reference coat- and claws-specific data about physiologic conditions and, possibly, information useful to detect reproductive disturbances or pathologies related to pregnancy and post-partum, perinatology

and puberty. Moreover, these matrices could be also useful for detecting the diverse reproductive statuses (prepubertal, pubertal or gonadectomized) in male and female dogs and cats.

The results obtained about the use of hair and nails in human and veterinary medicine, however, demonstrated the high potentiality of these matrices to deepen the study of many endocrine aspects, not only about reproduction. Although the major interest in the use of hair and nails for hormonal studies in both human and veterinary medicine, some aspects still need to be better clarified. For instance, as reported in humans, the region of the body in which the hair/coat is collected could differently influence the extent of hormones accumulation. This factor should be even more considered when claws collection is concerned. Other than the different weight load between forelimbs and hindlimbs, indeed, also the different position of some claws should be considered, for example the dewclaws. In the present thesis, this possible confounding factor was limited by collecting coat samples always from the same area. About the use of claws, different protocols of collection were designed according to the reproductive phases (and age and habits of the enrolled subjects). Thus, in the study of perinatology, because all the puppies were kept indoor until weaning, in boxes with a smooth floor, the claws were supposed to undergo a limited process of consumption. Therefore, the claws of all the digits were collected and pooled for each puppy in each sampling time. On the opposite, in the study about puberty in which the claws were planned to be collected only once in dogs and cats, it was chosen to collect only dewclaws, according to a couple of reasons: firstly, the dewclaws are less prone to be lose/broken by the habit of scratching in cats, and walking in dogs; secondly, the claw-shedding could be less intense in the dewclaws than in the claws of all the other digits.

Thus, for a more complete study about the use of claws for the measurement of long-term hormonal changes, further studies are needed to assess possible similarities or differences according to the type of claws samples collection (dewclaws vs claws of other digits; forelimbs vs hindlimbs; etc). Moreover, although claws have been reported to be similar among animals, specific investigations on possible differences between dogs and cats are desirable. Other than this, a detailed study on the influence of the pigmentation on the hormonal incorporation seems to be necessary. In the pregnancy and post-partum project of the present PhD thesis, statistically significant differences were found between black-and-rust and brown-and-rust coats, although all the enrolled bitches belonged to a single breed. This finding is intriguing because the limited knowledge about this topic mostly refer to differences between light and dark coat in different animal species, whilst, as previously suggested in literature, also nuances of colors could have an influence on the hormonal accumulation in hair. The results of the present study, indeed, highlight that also little variations of color could influence the hormonal accumulation in the

coat of dogs. Unfortunately, the heterogeneity of coat colors collected in the two projects about puberty in dogs and cats, prevented the assessment of a similar influence on the coat collected only once in dogs and cats. However, this aspect needs to be better investigated in further studies in both species. About claws, instead, information about the possible influence of pigmentation on hormonal accumulation are lacking, and thus this topic deserves further scientific investigations.

Another aspect that should be clarified when coat is used as a matrix, is the role of the molt in both dogs and cats. Furthermore, also the coat length of the shaft (and therefore differences among breed within each species) should be investigated in relation to accumulation of hormones, as reported in humans.

For what concerns claws, the possible effect of weight bearing, walking and claw-shedding should be better investigated in dogs, while in cats more attention should be payed to the role of scratching and claw-shedding on the real extent and time-window of accumulation of hormones.

All these factors could, indeed, influence the incorporation of hormones. Clarifying timing, extent and factors affecting this phenomenon could lead to a more accurate interpretation of the obtained results. A partial overcome of these unsolved points could be obtained by the "shave-reshave" technique for coat (clip and re-clip for claws), that allows a better identification of the interval of accumulation. This approach was used in the pregnancy and post-partum project, in which the first coat sampling was considered as the starting point to assess the accumulation of hormones during the subsequent samplings. In perinatology, instead, the first claws collection allows a roughly timing of retrospective time of hormones accumulation. In fact, the time between the appearance of the claws in the fetus and the first collection was presumed to be dated at about 30 days of intrauterine life. However, to the best of the author's knowledge, at present, studies about the precise timing of development of claws during intrauterine life in dogs and cats are lacking. In the studies about perinatology, the time interval of 30 days was chosen to cope with specific phases of transition of the newborns, e.g. second half of intrauterine development to birth (whose possible accumulation of hormones was measured on samples collected at birth), birth to the end of the neonatal period/beginning of weaning (hormones measured on samples collected at 30 days after birth), and from the end of the neonatal period/beginning of weaning to the end of weaning (hormones measured on samples collected at 60 days after birth). At the second and third claws collection, claws regrowth was detectable by the slightly different color of the new tissue.

Beside a general discussion about the strengths and weaknesses of using coat and claws for long-term hormonal changes during long-lasting phases in companion animal reproduction, some specific considerations should be addressed to each one of the three project lines.

In the pregnancy and post-partum project line, the sample collections have proved to be easy and non-stressful for bitches. Indeed, the collection was performed in a very little range of time and in any case the bitches were not moved from the breeding facility, but, as well as the perinatology project, samples collections were performed in their familiar environment. This last factor, coupled to the fact of avoiding the stress of transportation, strongly reduced the distress. In all cases the bitches showed to be relaxed and calm, and allowed the collections. In this project line, some data about the monthly variations of cortisol (C) and dehydroepiandrosterone (DHEA) + dehydroepiandrosterone sulfate (DHEA-S), together expressed as DHEA(S), from mating until puppies weaning, were provided. The results showed higher C and DHEA(S) measured in the coat at 60 days post-partum than at 30 days of pregnancy. Thus, given the retrospective characteristic of the hormones measured in the coat, the results seem to suggest that the HPA axis activation was higher during the second month post-partum. This finding agrees with literature, in which a recent study reported higher C concentrations in blood of cats at 4 weeks of lactation, that is, most of the time, the moment of the peak. Unfortunately, the fact of stopping the coat collection at 60 days post-partum prevented the assessment of the exact time in which C and DHEA(S) return to the initial values and deserves further investigations. Because of the Covid-19 pandemic emergency rules, the work at the laboratory was strongly limited and data about sex steroids were postponed. Therefore, those data were not available for the present thesis. Although it could be interesting to perform the same study also in female cats, the contacted cat breeders were strongly less compliant than dog breeders and did not allow to enroll cats for this study.

For what regards perinatology, the collection of coat was reported to be not appropriate for the studies in newborn puppies, because of the need of shaving a large area in very small sized subjects, leading to risk of harmful or even life-threatening skin damage. Thus, we proposed claws as the matrix of choice for studies about long-term accumulation of hormones in newborn puppies. As previously briefly mentioned, despite the pigmentation of the claws, that in most of the cases became black already at 30 and/or 60 days of age, the recognition of the only re-grown claws was easily detected by the slightly different color in the newly regrown claws. Moreover, although the very small size of the claws in newborns, only the avascular tips was always collected. A last mention should be addressed to the complete compliance of breeders to clip and collect claws from newborn puppies. Most of the breeders, indeed, usually cut the claws of puppies after birth to prevent scratching to the mammary glands of the

bitches, possible prerequisite for infection development and creating painful conditions for the mothers, that could also lead to possible maternal behavior disturbances. The subsequent collection at 30 and 60 days of age was recognized by the breeders as a contribution to accustom the puppies to the human manipulation. About the results, significant trends of decrease of C and DHEA(S) were found from the sample collected at birth as compared to the following samplings at 30 and 60 days after birth, and between 30 and 60 days of age. Moreover, an effect of the type of birth was played on both hormones on the samples collected at birth, with higher concentrations observed in puppies born by vaginal delivery than in the ones born by caesarean section, suggesting a different HPA activation in puppies born by the two different types of birth. In the second study a significant decrease of E2 and T was seen from birth to 30 and 60 days of age, but not from 30 to 60 days of age. Greater T concentrations were found in males than females, with an interaction between sex and sampling time. The Apgar score was positively related to T concentrations in claws collected at birth, while the bodyweight was positively correlated with T concentrations, with an interaction among puppy sex, bodyweight and sampling time. Results indicated that there are greater E2 and T concentrations at birth compared with 30 and 60 days of age, and this could be the result of these prenatal steroids affecting fetal development. These findings add precious information regarding the perinatal physiology, a very delicate and intricate period reach of transitional phases (birth, weaning, etcetera). Further investigations are needed to explore this phase, and claws have proved to be a suitable matrix for these types of studies.

The most time-consuming project line of the present PhD thesis was certainly the one dedicated to puberty. In fact, the former aim was to follow dogs and cats from the age of weaning until puberty attainment. For cats the same problem of compliance from the breeders, as stated above, was soon evidenced, so that the study on cats was shifted to a more feasible, acceptable by cats' owners, investigation in which a single coat and dewclaws sample was collected during routine clinical examination of cats of known reproductive status (prepubertal, pubertal, gonadectomized), and analyzed for the two main sexual steroids: 17β-estradiol (E2) and testosterone (T). Other hormones, such as progesterone, C and DHEA(S) were planned to be assessed, but the laboratory could only provide results about E2 and T as preliminary results. The study provided evidence that both matrices were useful for sex steroids measurement, but coat provided more interesting results, suggesting more focused investigations about the reliability of using dewclaws for retrospective hormonal accumulation measurement in the cat. It was indeed observed that coat T was significantly different between pubertal males and females and between gonadectomized male and females. Only T coat concentrations were different between pubertal and prepubertal male cats. The results provided useful information for the

further investigations about the use of these matrices, collected only once, to distinguish prepubertal, pubertal and gonadectomized subjects within a sex, in the domestic cat. However, further implementation (higher number of cases, equal distribution of cases within each sex and within each reproductive status, better definition of each reproductive status, wider hormonal analyses) were reported to be requested for a more precise understanding of the results.

In the dog, instead, monthly collections of coats were performed on 12 dogs from weaning to puberty attainment. However, because of the Covid-19 pandemic rules limiting the work at the laboratory, the analyses were postponed to the Autumn 2020, preventing the use of data for the present thesis. However, likely the study performed in the cat, also in dogs a single coat and dewclaws collection from male and female dogs of known reproductive statuses (prepubertal, pubertal and gonadectomized), was performed during routine clinical examination. On these samples, the laboratory was able to analyze not only E2 and T, but also C and DHEA(S) only on coat, so that, also for this study, preliminary data were provided. The results confirmed the usefulness of the dogs' coat as a matrix for the measurement of long-term retrospective accumulation of hormones, even though some weaknesses related to the single sampling arose and deserve further investigations. Therefore, the obtained results must be considered only preliminary and cautiously interpreted. In prepubertal and pubertal dogs, only T coat concentrations were significantly higher in males than females. In pubertal dogs, DHEA(S) and C coat concentrations were higher in males than females. Although higher T and DHEA(S) in males are not surprising, the higher coat C concentrations in males than females seems to suggest that the pubertal state could be more stressful for males.

The assessment of possible differences about the long-term concentrations of hormones in coat among the three reproductive statuses within each sex, showed a significantly lower DHEA(S) concentration in pubertal than prepubertal and gonadectomized female dogs. In males, T coat concentrations were higher in pubertal than prepubertal dogs, and C coat concentrations were higher in pubertal than prepubertal and gonadectomized dogs. Data seem to suggest that the pubertal status in male dogs entails not only the expectable higher exposition to T in comparison to the prepubertal stage, but also that pubertal status leads to a higher activation of the HPA axis and, in turn, C secretion that accumulates in the coat.

Taken all the results obtained on coat and claws used for the retrospective hormones accumulation measurement, some strengths and weaknesses were evidenced, and could be summarized as follows:

Strengths

- Noninvasive collection in newborn young, adult dogs and cats, and in pregnant-lactating bitches
- Full compliance by dog breeders and by dog and cat owners
- Easy collection, transport and storage
- Repeatability of collection allow the shave-reshave or clip-reclip program collection
- Analyses performable on even very small amount of sample
- Useful for long-term longitudinal studies, because of limitation of samplings

Weaknesses

- Mostly absence of reference data for hormones measured on these new matrices
- Differences of characteristics between coat and claws within and between dogs and cats
- Lack of exact information about the growth rate of coat and claws in dogs and cats
- Lack of information about possible effect of claw-shedding in cats on hormonal incorporation and measurement
- Conflicting results about the effect of coat color in dogs and lack of similar information in cats
- Lack of information about the role of body region collection on coat in companion animals, and lack of information about possible differences between claws collected by different legs or even digits
- Difficult interpretation of factors affecting the measurement of hormones in a single collection of coat and claws

Other than these weaknesses, also the enrollment of animals should be more carefully designed.
In the study on cats about the single coat and claws sampling, a higher number of subjects balanced for sex and for each reproductive status is needed. More detailed information will be necessary to detect the real prepubertal status and, in pubertal females, also the time elapsing between the beginning of the breeding season and the time of collection should be better scheduled. Moreover, the analysis of all the hormones studied in the dogs should be performed to obtain a more complete information.
In the dog, data about the samplings from weaning to puberty attainment will probably be helpful in better understanding the results obtained in the present study. However, also in dogs, a higher number of subjects balanced for sex and for each reproductive status is needed, and more detailed information about the real prepubertal status will be necessary.
Nonetheless, the obtained results provide useful information for the further investigations on the use of these matrices, collectable repeatedly or even only once, to study some reproductive phases in dogs and cats that still remain incompletely explored and that deserve scientific and practical interest.

The last comment on the use of coat and claws as matrices of study is the remarkable easy storage. Samples were indeed stored in paper envelopes at room temperature, avoiding the use of particular instruments and their related costs, allowing also a simple transportation.

A final mention is for the use of fetal fluids as a noninvasive matrix of study in perinatology. Fetal fluids have been reported as a matrix collectable without invasiveness in dogs submitted to caesarean section, with no risks for newborns. In the last decades, results have been reported from studies performed in dogs and cats. Those studies improved some knowledge about the fetal fluids composition in companion animals and contributed to deepen the still scarce information about companion animal perinatology. In the present PhD thesis, an additional study on fetal fluids was performed, to add new data about some aspects of perinatal metabolism already started with the study of insulin-growth-factor-I (IGF-I) and non-esterified fatty acids (NEFA) in fetal fluids. The results indicated a relation between fetal fluids IGF-I and NEFA fetal fluids concentrations and breed body-size. Therefore, a study was performed to deepen the possible effect played by breed body-size on amniotic leptin concentrations in dogs. The results about leptin amniotic concentrations in relation to canine breed body-size showed that leptin amniotic concentrations were higher in small- as compared to large-sized breeds, evidencing that, when the metabolism is studied, the breed body-size should be kept into consideration, as previously suggested for other factors such as IGF-I and NEFA. The limitation of using fetal fluids in companion animals is due to the still limited application of fetal fluids collection during pregnancy and its repeatability. Therefore, at present, fetal fluids proved to be a useful and interesting matrix, in which the main limitation is to allow only one-time measurement that, however, could be considered as a result of a cumulative action of the fetal and maternal compartments.

CONCLUSIONS

In conclusion, the results reported in the present PhD thesis demonstrated the usefulness of matrices collectable without invasiveness for the study of some long-lasting reproductive phases in companion animals.

Even if the enrollment of animals, the samples collection, and the results obtained were partially impaired by the sudden Covid-19 pandemic and consequent emergency lockdown rules, interesting and satisfactory data were provided.

In the study about pregnancy and post-partum, the results about coat C and DHEA(S) changes from mating to 60 days post-partum evidenced that, as recently reported for the cat, also for the dog maternity could be considered as a challenge and that focused investigations are desirable for a better selection of bitches on the base of their maternal attitude.

In the study about perinatology, the usefulness of claws as matrices for the longitudinal study of C, DHEA(S), E2 and T changes from birth to 60 days of age was evidenced, and results were shown on two papers on international peer reviewed, high IF and Q1 journals. In addition, results about leptin amniotic concentrations in newborns also provided results published on an international peer reviewed, high IF and Q1 journal.

The project line that was more affected by the Covid-19 pandemic and emergency rules was the one about puberty. However, some preliminary data about the use of coat in dogs, and coat and claws in cats, collected only once to assess the concentrations of several hormones in relation to the known reproductive status, evidenced that those matrices are promising tools for such kind of studies. However, more detailed technical information about the use of these matrices should to be investigated, coupled to a more precise selection of the animals.

LIST OF PUBLICATIONS AND CONFERENCES

• Scientific publications in the field of reproduction - international peer reviewed journals:

Fusi J, Comin A, Faustini M, Prandi A, Veronesi MC. (2018) The usefulness of claws collected without invasiveness for cortisol and dehydroepiandrosterone (sulfate) monitoring in healthy newborn puppies after birth. Theriogenology 122:137-143. doi: 10.1016/j.theriogenology.2018.09.016

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