Veterinarni Medicina, 60, 2015 (10): 567–577 *Original Paper*

doi: 10.17221/8495-VETMED

Plasma ACTH, α-MSH and cortisol variations in the dog during the oestrous cycle in different photoperiods

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ABSTRACT: The hypothalamic-pituitary-adrenal axis (HPA) is a complex system regulated by multiple factors. Sexual dimorphism of this axis has been described in different species under physiological conditions and it has been proposed that sexual hormones could have an effect on it. There are only a few reports about sex-linked variations in HPA axis hormones in the dog. Thus, studying the impact of sexual hormones on the HPA axis would broaden the knowledge about its function in this species. Therefore, the objective of this study was to determine whether there are variations in HPA plasma hormones (ACTH, alfa-melanocyte-stimulating hormone [α-MSH] and cortisol) according to the sex and photoperiod (positive or negative photoperiod were considered when the duration of the light hours of the day was more than 12 or less than 12, respectively) under basal conditions (like anoestrus) and throughout the oestrous cycle in the female dog. The population under study consisted of 11 intact female and 14 intact male dogs. Under basal conditions neither ACTH nor α-MSH concentrations showed differences between sexes and different photoperiods. Cortisol showed greater values in the negative photoperiod than in the positive, both in females and males (P = 0.03 and P = 0.015, respectively). Throughout the oestrous cycle, all the studied hormones showed variations (P < 0.0001). The greatest concentrations of ACTH were observed at proestrus, while α-MSH and cortisol showed their greatest concentrations at oestrus. The three hormones decreased in diestrus**.** ACTH and cortisol concentrations were higher in the negative photoperiod (P = 0.04 and P < 0.0001, respectively), while α-MSH concentrations were higher in the positive photoperiod (P = 0.012). In the group of females oestradiol and progesterone correlated with ACTH (r = 0.75, P < 0.0001; r = 0.34, P < 0.01, respectively), α-MSH (r = 0.49, P < 0.0001; r = 0.52, P < 0.0001, respectively) and cortisol (r = 0.33, P < 0.01; r = 0.5, P < 0.0001, respectively). These results show that in females, HPA axis hormones vary during the oestrous cycle in relation to oestradiol and progesterone fluctuations. The ACTH, α-MSH and cortisol concentrations also showed differences between photoperiods in females, but only cortisol did so in males. These findings suggest that sexual hormones could have an effect on the HPA axis. Further research needs to be done to fully understand this interaction and the mechanisms involved.

Keywords: corticotroph cell; adrenal gland; ACTH; oestrous cycle; sexual dimorphism

Sexual dimorphism of the HPA axis has been described in various species (Gaillard and Spinedi 1998; Seale et al. 2005; Romeo 2010). Most studies have been performed in rats, so the present work uses this model for a comparison of the results. However, it is important to consider that adrenal steroidogenesis shows differences between species (Beuschlein et al. 2012). In rodents, after synthesis of pregnenolone, subsequent steps lead to the synthesis of corticosterone (the main glucocorticoid in these animals); while in the dog, the presence of 17α-hydroxylase leads to the synthesis of cortisol (Rosol et al. 2001).

Atkinson and Waddell (1997) reported differences in plasma corticosterone between female rats at proestrus and males, suggesting that oestradiol

Supported by the UBACyT, University of Buenos Aires, Argentina (Grants No. V006 and No. 20020100100246).

doi: 10.17221/8495-VETMED

(E_2) is responsible for this finding. In sheep, cortisol concentration post-ACTH in ewes is greater than in rams, and these differences between sexes disappear when comparing gonadectomised males and females (van Lier et al. 2003a). Pessina et al. (2009) reported a sex effect in the diagnostic tests for Cushing's syndrome in healthy dogs, as the cortisol-induced response to ACTH stimulation and dexamethasone inhibition were greater (e.g. increased and decreased cortisol concentrations, respectively) in females than in males.

Although several reports on the effect of E_2 on the HPA axis performed in cell cultures and animal models (rats, sheep) are available (McCormick et al. 1998; Ochedalski et al. 2007; Ogura et al. 2008; Kageyama and Suda 2009; Weiser and Handa 2009; Handa and Weiser 2014; van Lier et al. 2014), the mechanisms have not yet been fully elucidated. In general, it has been proposed that E_2 could exert a stimulatory effect on the hypothalamus, the corticotroph cells or the adrenal gland cortex (by stimulating CRH secretion or inhibiting glucocorticoid feedback, or stimulating ACTH synthesis or cortisol synthesis/secretion) (Burgess and Handa 1993; Carey et al. 1995; McCormick et al. 1998; Lo et al. 2000; van Lier et al. 2003b; Ochedalski et al. 2007; Kageyama and Suda 2009; Weiser and Handa 2009; Handa and Weiser 2014; Panagiotakopoulos and Neigh 2014).

Furthermore, data about the influence of progesterone (P_4) on the HPA axis are limited. Although it has been found that both in women and sheep in advanced pregnancy, plasma ACTH and cortisol are increased, the effect of P_4 on the previously mentioned hormones could not be determined (Goland et al. 1994; Keller-Wood 1998; Keller-Wood and Wood 2001). Moreover, there were no variations in ACTH and alfa-melanocyte-stimulating hormone [α-MSH] concentrations in bitches that were treated with synthetic P_4 (Beijerink et al. 2007).

With regard to androgens, it has been proposed that they could exert an inhibitory action on the HPA axis, as in gonadectomised adult male rats testosterone administration decreased corticosterone concentrations (McCormick et al. 2002; Lund et al. 2004; Handa et al. 2009; Evuarherhe et al. 2009).

It has also been suggested that the photoperiod may affect the HPA axis, as differences in cortisol concentrations over the course of the year were reported in various species (Romero 2002). Furthermore, differences in the response to ACTH stimulation according to the season were described in sheep (van Lier et al. 2003a). In dogs, no reports were found on the seasonal effect of hormone concentrations on the HPA axis.

Since there are only a small number of reports about sex-linked and seasonal variations in HPA axis hormones in the dog, studying the impact of these factors on the HPA axis could broaden the knowledge about its function in this species. Also, it could open up a way to studying the relevance of these findings in HPA axis pathologies, as has been suggested in studies performed in humans, rats and dogs (Solomon and Herman 2009; Gallelli et al. 2010; Goel et al. 2014). In the present study it was hypothesised that the HPA axis hormone profiles (ACTH, α-MSH and cortisol) are affected by sex, photoperiod and the phases of the oestrous cycle.

MATERIAL AND METHODS

Animals. The study was approved by the Ethics Committee of the Faculty of Veterinary Sciences and the Grants Committee (CICUAL, UBACyT V006 y 20020100100246) of the University of Buenos Aires, and complied with the current national laws and international regulations on the use of animals in clinical studies. The study was developed in Buenos Aires City, Argentina (latitude 34°30'S, longitude: 58°26'W, altitude: 25 m.a.s.l, 21st September is spring equinox and 21st December is summer solstice; autumn equinox is the 21st March and winter solstice is the 21st June). Photoperiods were defined as follows: positive photoperiod (Ph+) was considered when the duration of the light hours of the day was more than 12 (mean 14 h 15 min), while negative photoperiod (Ph–) was considered when the duration of the light hours was less than 12 (mean 10 h 04 min). Thus, the period from the second fortnight of November to the first fortnight of February corresponded to Ph+, while the period from the first fortnight of May to the second fortnight of July corresponded to Ph–. These periods were selected and defined taking into account the records of normal appearance of oestrous cycles of the females from the vivarium of the Faculty of Veterinary Sciences, University of Buenos Aires.

Two studies were performed; all animals included in both studies were healthy with no previous pathologies and belonged to the vivarium of the previously mentioned institution. The animals spend the

doi: 10.17221/8495-VETMED

day outdoors, limited by a perimeter fence, and at night they are taken into individual indoor kennels.

Study 1: Hormone concentrations (basal conditions) in dogs of different sex in negative and positive photoperiods. To study the effect of photoperiod (negative and positive) and sex on ACTH, α-MSH and cortisol, intact females in anoestrus (n = 11) (defined as the phase of the cycle characterised by the lack of clear evidence of ovarian activity, with P_4 concentrations below 1–2 ng/ml) (Concannon 2011) and intact males (n = 14), were studied**.** We defined these animals as being in 'basal condition' for the purposes of comparison with the 'oestrous cycle condition' (see Study 2). The selected animals comprised the intact females of Study 2 and 14 beagle intact males. The median age of the whole population was six year (5–10 years). The blood collection in males and females in anoestrus was performed at the same time in both photoperiods.

Study 2: Hormone concentrations during the oestrous cycle in negative and positive photoperiods. To study the hormone concentrations during the oestrous cycle in negative and positive photoperiods, 11 intact females were used. Animals comprised 11 beagles, aged 5–9 years (median = six years). Following the vivarium records, it was known that the females had their oestrous cycles between May and August and repeated them between December and February. Taking into consideration this information, the blood collection in anoestrus was performed before the expected beginning of proestrus, and from then on, blood was collected once a day every 24 h (see Blood sample collection). In order to determine the phases of oestrous cycle, cytological analyses (Shorr's staining modified by Schutte) and vaginoscopy every 48 h (Schutte 1967a; Schutte 1967b; Schutte 1967c) were performed. The P_4 and E_2 serum concentrations were also determined (Schutte 1967c; Concannon 1987; Bouchard et al. 1991). Thus, the blood analyses performed in this study included, anoestrus (AE) (P_4 concentrations < 6.36 nmol/l and E_2 concentrations < 36.7pmol/l, corresponding to 21 ± 5 days before the beginning of vaginal bleeding), end of proestrus (PE) (P_4 concentrations < 6.36 nmol/l and E_2 concentrations > 184 pmol/l, corresponding to 8 ± 2 days after the beginning of vaginal bleeding), beginning of oestrus (E) (P_4 concentrations between 6.36 nmol/l and 22.8 nmol/l, corresponding to 10 ± 3 days after the beginning of vaginal bleeding) and diestrus (DE) (P_4 concentrations > 60 nmol/l, corresponding to 21 ± 3 days after the end of E). The proestrus and oestrus correspond to the follicular phase and diestrus, to the luteal phase.

Blood sample collection. Blood samples were obtained every 24 h, at the same time of the day (8 a.m.). One blood sample was collected from each dog every day. Samples obtained to measure ACTH and α-MSH were collected into refrigerated plastic tubes with EDTA and aprotinin. They were immediately centrifuged and frozen at –80 °C until hormonal assays were performed. Samples for cortisol, E_2, P_4 and testosterone (T) determinations were collected in glass tubes, centrifuged and stored at –80 °C until the assay was performed.

Hormone assay. All of the following methods were previously validated for dogs (Ravivarapu et al. 2000; Gallelli et al. 2010; Cabrera Blatter et al. 2011; Ithurralde et al. 2013).

Serum oestradiol concentrations were measured with a double-antibody radioimmunoassay (RIA) using commercially available kits (oestradiol double antibody, KE2D; DPAC). The intra-assay coefficient of variation was 6.3% and the inter-assay coefficient of variation was 9.3%. The assay sensitivity was 18 pmol/l.

Serum progesterone concentrations were measured using a direct solid-phase commercial RIA (Coat-A-Count; DPC, Los Angeles, CA, USA). The intra-assay coefficient of variation was 6.3% and the inter-assay coefficient of variation was 7.9%. The assay sensitivity was 0.32 nmol/l.

Serum testosterone concentrations were measured using solid-phase RIA (Diagnostic Products, Los Angeles, CA, USA). The intra-assay and inter-assay coefficients of variation were 7.7% and 8.5%, respectively. The assay sensitivity was 0.069 nmol/l.

Plasma ACTH concentrations were measured using an enzyme immunoassay (EIA), with a commercial kit (Alpco Diagnostics). The intra-assay and inter-assay coefficients of variation were 6.7% and 7%, respectively. The assay sensitivity was 1.1 pmol/l.

Plasma α-MSH concentrations were measured using RIA (Euro-Diagnostica AB, Malmo, Sweden). The intra-assay and inter-assay coefficients of variation were 3% and 4%, respectively. The assay sensitivity was 0.6 pmol/l.

Serum cortisol concentrations were measured using RIA (DPC Corporation, San Diego, CA, USA). The intra-assay and inter-assay coefficients

doi: 10.17221/8495-VETMED

of variation were 5% and 8.5%, respectively. The assay sensitivity was 13.8 nmol/l.

Statistical analysis. Hormone concentrations in the different phases of the oestrous cycle in each photoperiod and between photoperiods were analysed by one-way ANOVA, followed by the Tukey-Kramer test. In order to study the interactions between the phases of the oestrous cycle and the photoperiod (Study 2) or the sex and the photoperiod (Study 1), a 2-way ANOVA followed by Bonferroni test was performed (Graph Pad 5, USA). Pearson's correlation was calculated to study the relationships between hormone concentrations. Values are expressed as mean ± SEM. Differences were considered significant when P values were < 0.05

RESULTS

Study 1. Hormone concentrations (basal conditions) in dogs of different sex in negative and positive photoperiods

Oestradiol, progesterone and testosterone serum concentrations in the serum. Under 'basal conditions', E_2 and T in females and males were affected by sex (P = 0.0002) but not by photoperiod (Table 1). Oestradiol was higher in females than in males (P = 0.04), and T was higher in males than in females (P = 0.0011). Progesterone was not affected by sex or photoperiod (Table 1).

Concentrations of plasma ACTH and α-MSH, and serum cortisol. Concentrations of ACTH and α-MSH (Table 2) were not affected by photoperiod and sex (there were no statistical differences between females and males). Photoperiod affected cortisol concentrations (P = 0.04), being higher in Ph– than in Ph+ in both females (P = 0.03) and males (P = 0.015) (Table 2).

Study 2. Hormone concentrations during the oestrous cycle in negative and positive photoperiods

Oestradiol and progesterone serum concentrations in the serum. Oestradiol concentrations (Figure 1) were affected by the photoperiod (P = 0.011) with larger values in Ph–, and as was expected, by the phase of the oestrous cycle (P < 0.0001; F: 47.26). E_2 reached its peak at PE and then decreased at E and DE in both photoperiods (Figure 1). Differences between photoperiods were observed at PE (P < 0.01). A statistical interaction between the photoperiod and the oestrous cycle was not found.

Progesterone was not affected by the photoperiod (P = 0.9) but it was affected by the phase of the oestrous cycle (P < 0.0001; F: 354.9). Its concentration increased progressively and reached its peak at DE as expected (Figure 1), in an almost identical manner in both photoperiods.

ACTH and α-MSH plasma concentrations and cortisol serum concentrations. The ACTH concentrations showed differences between the phases of the oestrous cycle (P < 0.000; F: 18.54) and between photoperiods (P = 0.04). An interaction between both factors was found (P = 0.0004). This hormone's profile in both photoperiods resembled those of E_2. ACTH reached its greatest concentrations at PE (P < 0.001 vs. AE), before subsequently decreasing at E (E vs. AE; P < 0.001 Ph–, P < 0.01 Ph+), but its concentration was stably maintained at DE, and remained higher than AE (DE vs. AE; P < 0.01Ph– , P < 0.001 Ph+) (Figure 2). Differences between photoperiods were observed only in PE, showing greater concentrations in Ph– compared to Ph+ (P < 0.001).

The α-MSH (Figure 3) concentrations showed differences between the phases of the oestrous cycle (P < 0.0001, F: 22.04) and between photo-

Table 1. Oestradiol, progesterone and testosterone concentrations in intact anoestrous females (F) and intact males (M) in negative and positive photoperiods

Hormones	Positive photoperiod		Negative photoperiod	
	F	M	F	M
E_2 (pmol/l)	33.8 ± 1[a]	29.3 ± 0.7[b]	31.5 ± 1.1[a]	27 ± 0.7[b]
P_4 (nmol/l)	1.6 ± 0.1	1.3 ± 0.12	1.5 ± 0.2	1.34 ± 0.15
T (nmol/l)	0.14 ± 0.02[x]	17 ± 1.7[y]	0.12 ± 0.01[x]	18 ± 1.6[y]

Values are expressed as mean ± SEM; E_2 = oestradiol, P_4 = progesterone, T = testosterone

a vs. b = differences between females and males in oestradiol concentrations in both photoperiods (P = 0.04)

x vs. y = differences between females and males in testosterone concentrations in both photoperiods (P = 0.0011)

doi: 10.17221/8495-VETMED

Table 2. Hormone concentrations in intact anoestrous females (F) and intact males (M) in negative and positive photoperiods

Hormones	Positive photoperiod		Negative photoperiod	
	F	M	F	M
ACTH (pmol/l)	3 ± 0.07	2.7 ± 0.08	2.9 ± 0.09	2.8 ± 0.09
α-MSH (pmol/l)	5.9 ± 0.1	6.5 ± 0.2	5.9 ± 0.1	6.2 ± 0.2
Cortisol^{+} (nmol/l)	37 ± 5^{a}	34 ± 4^{b}	48 ± 4	51 ± 5

Values are expressed as mean ± SEM

$^{+}$the effect of the photoperiod on cortisol concentrations ($P = 0.04$)

$^{a}P = 0.03$ and $^{b}P = 0.015$ indicate differences in cortisol concentrations between photoperiods in females and males, respectively

periods ($P = 0.012$). An interaction between both factors could be determined ($P < 0.0001$). In both photoperiods α-MSH concentrations increased at PE, reached their greatest concentrations in E, before diminishing at DE, but remaining higher than AE ($P < 0.001$). However, the decrease between E and DE was more pronounced in Ph+. Differences between photoperiods were observed only at E, showing greater concentrations in Ph+ than in Ph– ($P < 0.001$).

The cortisol concentrations (Figure 4) showed differences between the phases of the oestrous cycle ($P < 0.0001$, F: 19.1) and between photoperiods ($P < 0.0001$). Also, an interaction between both factors was determined ($P = 0.02$). In Ph+, it increased linearly, reached its maximum concentration at E and was stably maintained at DE. In Ph– cortisol reached its greatest concentration in E and decreased in DE, remaining higher than in AE and PE (Figure 4). Ph– showed greater cortisol concentrations than Ph+ both in E ($P = 0.0001$) and DE ($P < 0.05$).

Correlation analysis. In the group of intact females, during the oestrous cycle E_2 correlated with ACTH ($r = 0.75$, $P < 0.0001$), α-MSH ($r = 0.49$, $P < 0.0001$) and cortisol ($r = 0.33$, $P < 0.01$). There was also a correlation between P_4, ACTH ($r = 0.34$, $P < 0.01$), α-MSH ($r = 0.52$, $P < 0.0001$) and cortisol ($r = 0.5$, $P < 0.0001$).

Under the 'basal conditions' there was no correlation between the studied hormones in either females or males.

DISCUSSION

In this study it was demonstrated that sex and photoperiod did not affect the concentrations of ACTH and α-MSH under the 'basal conditions'. Cortisol was the only hormone that showed differences between photoperiods in both sexes, although differences between males and females were not found.

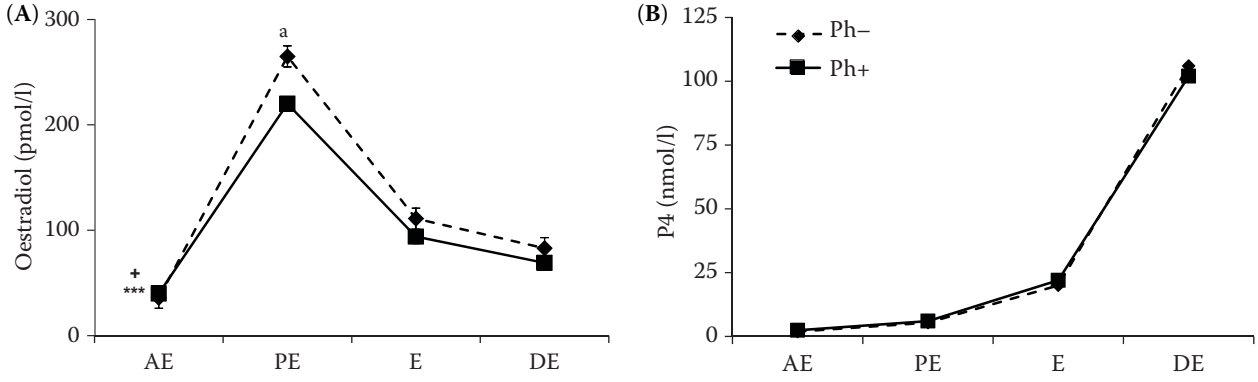

Figure1. Oestradiol (**A**) and progesterone (**B**) concentrations during the oestrous cycle in the female dog in negative and positive photoperiods. *** indicates the effect of the phase of the oestrous cycle on E_2 and P_4 concentrations ($P < 0.0001$). In Figure 1A (E_2), the letter "a" indicates significant differences between photoperiods at PE ($P < 0.01$). The effect of the photoperiod and the interaction with the phase of the oestrous cycle is indicated as "+" ($P = 0.011$)

doi: 10.17221/8495-VETMED

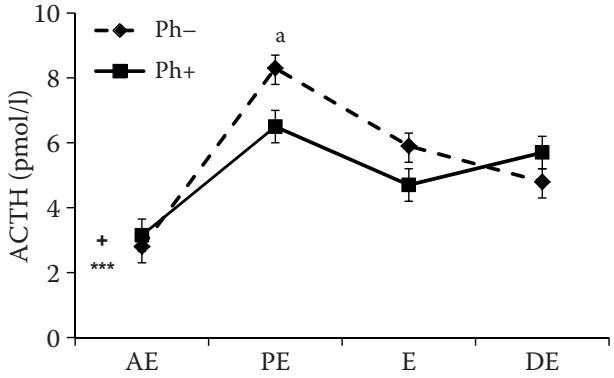

Figure 2. ACTH concentrations during the oestrous cycle in the female dog in negative and positive photoperiods. *** indicates the effect of the phase of the oestrous cycle on ACTH concentrations ($P < 0.0001$). The letter "a" indicates significant differences between photoperiods at PE ($P < 0.001$). The effect of the photoperiod ($P = 0.04$) and the interaction with the phase of the oestrous cycle is indicated as "+" ($P = 0.0004$)

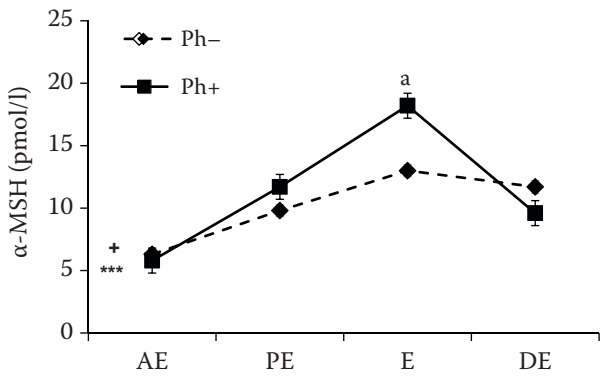

Figure 3. α-MSH concentrations during the oestrous cycle in the female dog in negative and positive photoperiods. *** indicates the effect of the phase of the oestrous cycle on α-MSH concentrations ($P < 0.0001$). The letter "a" indicates significant differences between photoperiods at E ($P < 0.0001$). The effect of the photoperiod ($P = 0.012$) and the interaction with the phase of the oestrous cycle is indicated as "+" ($P < 0.0001$)

Androgens have been described to inhibit the adrenal axis in other species (McCormick et al. 2002). However, in this study ACTH, α-MSH and cortisol concentrations did not show differences between males and females. No correlation with testosterone, at least under 'basal conditions', and HPA hormones was found. We did not evaluate the previously mentioned hormones during reproductive activity or in times of aggressive behaviour in males.

In contrast, in females ACTH, α-MSH and cortisol concentrations showed differences between photoperiods and phases of the oestrous cycle. These findings suggest that there could be an influence of both E_2 and P_4 on HPA hormones during the oestrous cycle as was shown in the Study 2.

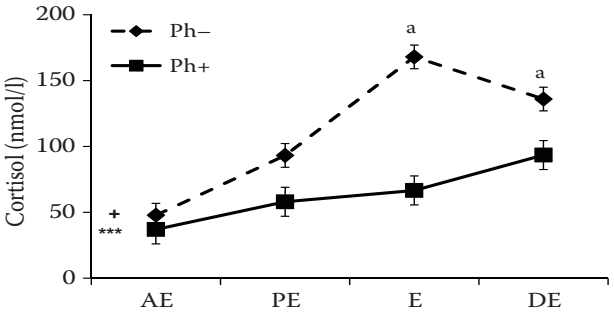

Figure 4. Cortisol concentrations during the oestrous cycle in the female dog in negative and positive photoperiods. *** indicates the effect of the phase of the oestrous cycle on cortisol concentrations ($P < 0.0001$). The letters indicate significant differences between photoperiods at E ($P < 0.001$) and DE ($P < 0.05$). The effect of the photoperiod ($P < 0.0001$) and the interaction with the phase of the oestrous cycle is indicated as "+" ($P = 0.02$)

Both sexual hormone profiles were similar to those already described in dogs (Rota et al. 2007). The concentrations of ACTH, α-MSH and cortisol fluctuate during the oestrous cycle, following the variation of E_2 and P_4. The changes in the levels of these hormones correlate and suggest that HPA axis activity may vary across the oestrous cycle in female dogs due to the influence of gonadal steroids. The effect of gonadal steroids has been reviewed recently (Handa and Weiser 2014). It is possible that variations in E_2 concentrations during the oestrous cycle, and particularly at PE (when maximum concentrations are reached), have an impact on the HPA axis, stimulating the synthesis and/or secretion of ACTH by means of different molecular pathways (Altemus et al. 2001; Hall et al. 2001; Labeur et al. 2010). Moreover, the drop in E_2 concentrations after PE is accompanied by a drop in ACTH concentrations, suggesting that the stimulatory effect of E_2 on corticotroph cells begins to diminish. In studies conducted in rat models and cell culture, it has been demonstrated that E_2 increases POMC mRNA synthesis (Ochedalski et al. 2007) and acts upon the paraventricular nucleus stimulating the release of the corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) (Greer et al. 1986; Ogura et al. 2008; Panagiotakopoulos and Neigh

doi: 10.17221/8495-VETMED

2014). These mechanisms may explain the observed variations in ACTH and its relation with E_2 during the oestrous cycle in female dogs, although this remains to be studied in this species.

P_4 might have a different effect over ACTH synthesis and secretion, depending on its physiological concentrations. It has been demonstrated in a rat model that P_4 inhibits the sensitising effects of oestrogen on ACTH, as the ACTH levels decreased with increasing amounts of progesterone in the oestrous and diestrus phases (Viau and Meaney 1991; Handa and Weiser 2014; Panagiotakopoulos and Neigh 2014). Similar changes were observed in our study. Carey et al. (1995) proposed that P_4 may have different effects on the HPA axis in the rat (stimulatory and inhibitory) by means of different mechanisms. Taking into account these studies performed in rats and the observations of the present study in dogs, we hypothesise that P_4 could stimulate ACTH synthesis at the beginning of the oestrous cycle (explaining the correlation between both hormones), and inhibit it when it reaches its greatest concentrations. This hypothesis remains to be tested in the dog.

In dogs, Beijerink et al. (2007) did not find changes in ACTH plasma concentrations in bitches treated with medroxiprogesterone, but found a lower response to CRF stimulation after the drug had been administered for a long time. The differences with this study could be explained by a differential effect between synthetic and native P_4 (related to chemical structure or physiological concentrations vs. therapeutic doses).

The α-MSH showed a similar profile to ACTH and also correlated with both sexual hormones. However, α-MSH reached its maximum concentrations at E and began to decrease during the DE. There are no reports about the possible impact of E_2 or P_4 on α-MSH synthesis. Considering the fact that E_2 may stimulate POMC synthesis (Ochedalski et al. 2007) and taking into account the fact that ACTH and α-MSH are secreted equimolarly (Gallelli et al. 2010), it is possible that the effect of E_2 may be the same for both hormones. The different profiles of ACTH and α-MSH observed after PE remain to be evaluated**.** One hypothesis could be that E_2 may have different mechanisms of action according to the cell type (melanotrope or corticotrope cell) (Tanaka 2003; Olah et al. 2009).

The interaction between the HPA axis and the gonadal axis, as well as the inhibitory effect of cortisol on gonadotrophic hormones and gonadal hormone synthesis has been widely studied (Hayashi and Moberg 1990; Oakley et al. 2009). Furthermore, adrenal steroidogenesis has been shown to be modified by sex steroids, as cholesterol conversion to pregnenolone is stimulated by oestradiol (a step of glucocorticoid synthesis common to rodents, humans and dogs) (Nowak et al. 1995; Rosol et al. 2001; Beuschlein et al. 2012). Our results are in agreement with Atkinson and Waddell (1997), who observed variations in corticosterone concentrations during the oestrous cycle in rats. In our study we also found significant differences in cortisol concentrations throughout the studied phases and this hormone correlated both with E_2 and P_4. On the one hand, cortisol is expected to rise in response to ACTH; on the other hand, it is possible that E_2 in female dogs has a regulatory effect over the fascicular zone of the adrenal cortex, as in other species (Viau and Meaney 1991; van Lier et al. 2003b). In post-menopausal women (Fonseca et al. 2001) and in ovariectomised rats (Lo et al. 2000) cortisol and corticosterone, respectively, increased after the administration of synthetic oestrogen. Moreover both in sheep and rats, ERα has been identified in the adrenal gland (Cutler et al. 1978) and recently our group has identified this receptor in the adrenal gland of the dog (Gallelli et al. 2012). Thus, it is possible that the increase in cortisol at oestrus may be a result of the combined effect of ACTH and E_2. Therefore, once cortisol synthesis is stimulated by the mentioned hormones at PE, its greatest concentrations would be detected in E. At DE, cortisol decreases as does ACTH. The role of P_4 on the adrenal cortex is controversial and not yet fully understood (Keller-Wood and Wood 2001).

Under 'basal conditions', only cortisol showed differences between photoperiods and was higher in Ph– than in Ph+. During the oestrous cycle, except for P_4, all the studied hormones showed differences between photoperiods, and the interaction between photoperiod, sexual and HPA hormones would be the determining factor in the observed differences. E_2, ACTH and cortisol concentrations were higher in Ph–, while α-MSH concentrations were higher in the Ph+.

Differences in hormonal concentrations between photoperiods have been described in the horse and the deer (Ingram et al. 1999; Donaldson et al. 2005), but not in the dog. Higher concentrations of ACTH in Ph– have been reported in different species and attributed to climatic stress, metabolic

doi: 10.17221/8495-VETMED

adaptations and reproductive function (Myers et al. 2010; Cordero et al. 2012).

The α-MSH behaviour between photoperiods differs from what has been described in the horse and other species, which show higher concentrations in Ph– (Cordero et al. 2012). These differences could be related to species-specific characteristics.

As previously mentioned, cortisol was the only hormone that showed differences between photoperiods both under the 'basal conditions' (in males and females) and during the oestrous cycle in females. The higher concentrations of cortisol at Ph– could be related either to the fewer light hours of the day during this photoperiod, or to climatic stress or metabolic adaptation, as has been reported in other groups of animals (Romero 2002; Cordero et al. 2012). It is very important to take into account the possible impact of melatonin on the HPA and gonadal axes of the dog, as reports in other species have indicated that it can determine the observed differences (Blaszczyk et al. 2004; Filippa and Mohamed 2006). Unfortunately, melatonin could not be evaluated in the present study.

Finally, it is important to consider that in the present study we did not perform hormonal profiles at different times of the day; thus, the obtained results are limited to the studied condition.

CONCLUSION

These results show that there are no differences in the plasma concentrations of HPA hormones between sexes under basal conditions (at the time of the day that blood collections were performed). In contrast, ACTH, α-MSH and cortisol concentrations in the studied intact females show variations across the oestrous cycle (at the time of the day that blood collections were performed), suggesting that E_2 and P_4 variations during this cycle could affect them. Plasma ACTH, α-MSH and cortisol show differences between photoperiods in intact females, while in males only cortisol does. The effect of testosterone or androgens during reproductive activity in males is still to be elucidated.

Further research needs to be performed to fully understand the interaction between the HPA axis and sex hormones, and the mechanisms involved. Understanding the nature of sex differences in HPA axis function is important not only to arrive at a more complete knowledge of its physiology but also because dysregulation of this axis is associated with several diseases.

Acknowledgement

We wish to thank the personnel of the vivarium and members of the HEMV laboratory for their help in this work.

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Received: 2014–12–29
Accepted after corrections: 2015–08–27

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