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Immunohistochemical analysis of the hypothalamic-pituitary-adrenal axis in dogs: Sex-linked and seasonal variation

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ABSTRACT

This study evaluated sexual dimorphism and seasonal variations in corticotrophs and adrenal zona fasciculata in dogs, as well as the expression of oestrogen receptor alpha (ERα). An immunohistochemical analysis was conducted in pituitaries for ACTH and in adrenal glands for ERα and for the melanocortin-2-receptor (MC2R) in winter and summer. Double immunofluorescence was performed to identify ERα in corticotrophs. Females had a greater proportion of corticotrophs per field ($p < 0.01$), with a greater cellular area and optical density ($p < 0.001$) than males. Optical density of corticotrophs was greater in winter for both sexes ($p < 0.001$). In zona fasciculata, ERα and MC2R expression was greater in females ($p < 0.001$) and was greater in winter ($p < 0.001$). ERα was identified in corticotrophs. This study is the first to demonstrate ERα expression in corticotrophs and the adrenal cortex in dogs, providing evidence for sexual dimorphism and seasonal variations.

1. Introduction

Functional sexual dimorphism in the hypothalamus-pituitary-adrenal (HPA) axis has been described in several species, including sheep, rats and, monkeys (van Lier et al., 2014). The activation of this axis is suggested to be sex-dependent, and in rat models, females are considered to have a greater stress response than males (Iwasaki-Sekino et al., 2009; Larkin et al., 2010). Sex-associated differences in HPA axis response have also been observed under basal conditions in rats and humans (Viau et al., 2005, Goel et al., 2014; Handa and Weiser, 2014). Pessina et al. (2009) reported differences according to sex in the cortisol response to adrenocorticotropic hormone (ACTH) stimulation and dexamethasone inhibition in healthy dogs, being this response greater in female dogs than in male dogs. Also, recently our group has reported variations in ACTH and cortisol plasma concentrations during the oestrous cycle in the dog (Gallelli et al. 2015). Rhodes and Rubin (1999) postulated that in mammals the differences in HPA axis function (hormone production) between sexes could be a result of morphological dimorphism (cell size, shape or number). The morphological dimorphism of corticotroph cells has been described in vizcachas (*Lagostomus maximus*), where female vizcachas have smaller but more numerous corticotrophs than males (Filippa and Mohamed, 2006). Also, in a study on the American mink (*Mustela vison*), Vidal et al. (1995) reported a considerable increase in the size of the area of corticotroph cells coinciding with the onset of puberty in females but not males, suggesting that this process may be affected by gonadal steroids. Although there are numerous reports detailing the effects of oestradiol on the HPA axis, the mechanisms of the hormone's action on this axis have not been completely elucidated (Handa and Weiser, 2014). Mitchner et al. (1998) identified oestrogen receptor alpha (ERα) in the corticotroph cells of the anterior lobe and in melanotrophs of *pars* intermedia in rats. Similarly, this receptor was found in the adrenal cortex of rats and sheep (Cutler et al., 1978; van Lier et al., 2003). Through ERα, oestradiol could stimulate adrenal steroidogenesis or affect adrenal sensitivity to ACTH as it has been reported in rats and sheep (Figueiredo et al., 2007; van Lier et al., 2014).

Differences in the cellular area and distribution of corticotroph cells in the pituitary gland have been described in certain species in different photoperiods, suggesting that these characteristics may be sensitive to seasonal variation (Hira et al., 2001; Filippa and Mohamed, 2006). This result is similar to changes described in the adrenal gland of vizcacha in which the nuclear volume of the adrenal cortex also shows seasonal variation (Ribes et al., 1999). Seasonal differences in ACTH and cortisol plasma concentration have also been reported in different species (Ingram et al., 1999; Romero, 2002; Cordero et al., 2012; Gallelli et al., 2015).

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To our knowledge, no studies have described the sexual dimorphism in the HPA axis morphology in dogs or seasonally associated changes. Thus, the aim of this study was to analyse whether morphological differences in corticotrophs occur between male and female dogs through different seasons in a year, and determine if the ERα is expressed in these cells. Also, this study aimed to evaluate whether ERα is found in the adrenal zona fasciculata and if its expression and that of the ACTH receptor (melanocortin-2-receptor, MC2R) varied by sex and season. These results would provide new information about HPA axis' morphology and receptor expression and would broaden the knowledge of its -characteristics. Taking into consideration the model proposed by Rhodes and Rubin (1999) this data could open up a way to studying the relationship between morphological and functional sexual dimorphism in the dog.

2. Materials and methods

2.1. Population under study

This study was approved by the ethics committee of the School of Veterinary Sciences and grant committee (CICUAL, UBACyT V006 and 20020100100246) of the University of Buenos Aires. The study was conducted in the city of Buenos Aires, Argentina (latitude: 34°30'S, longitude: 58°26'W, altitude 25 m.a.s.l; December 21st is the summer solstice and June 21st is the winter solstice). During the summer, there are more than 12 h of light per day (average 14 h, 15 min) and the mean temperature is 22.3 °C. During the winter, there are fewer than 12 h of light per day (average 10 h 4 min) and the mean temperature is 12.5 °C.

The pituitary and adrenal glands were obtained from dogs that were euthanized for humanitarian motives following recommendation by a veterinarian (e.g., medullar trauma). All animals underwent clinical evaluation before euthanasia. At that moment dogs were not in pain (assessed by Glasgow pain scale). Dogs suspected of suffering from infectious diseases or diseases that may affect the HPA axis (oncological diseases) were excluded from the study as were dogs that received glucocorticoid treatment prior to euthanasia. Dogs diagnosed with the aforementioned diseases during necropsy were also excluded from the study.

In summer, 7 female and 7 male dogs, all non-neutered, were included in the study, while in winter 7 female and 6 male dogs, all non-neutered, were considered. During the summer, samples were obtained from 5 mixed and 2 Labrador Retriever females (mean age: 8 years; mean weight: 29 kg) as well as 5 mixed and 2 Boxer males (mean age: 8 years; mean weight: 29 kg). During the winter, samples were obtained from 4 mixed and 3 Golden Retriever females (mean age: 8 years; mean weight: 31 kg) as well as 4 mixed and 2 Doberman males (mean age: 7 years; mean weight: 32 kg).

2.2. Immunohistochemical analysis

2.2.1. Immunostaining of ACTH in pituitary gland

Pituitary glands were fixed in 4% buffered formol and embedded in paraffin. Sagittal sections (5 μm thick) were mounted on silanised slides and subsequently rehydrated by carrying them through xylol and graduated alcohols, and finally through phosphate buffered saline (PBS, 0.01 M, pH 7.4). Sections were incubated for 20 min in a 3% H_20_2 solution in distilled water to inhibit endogenous peroxidase activity. After washing with PBS, non-specific binding sites were blocked by incubating in normal goat serum for 10 min ("Immunoperoxidase secondary detection system," DAB 150, Millipore, Temecula, CA, USA). Sections were washed with PBS and then, in order to identify corticotrophs, they were incubated in a humidified chamber at room temperature for 2 h with the primary anti-ACTH antibody (mouse monoclonal, sc-52,980, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:400 dilution in PBS. This antibody has been raised against 1–24 amino acids, which are common to all mammals (Mol and Meij, 2008). After washing in PBS, the "Immunoperoxidase secondary detection system" kit was used (DAB 150, Millipore, Temecula, CA, USA). Using this system, sections were incubated for 10 min with the anti-mouse biotinylated antibody. After washing with PBS, sections were then incubated for 10 min with Streptavidin-conjugated horseradish peroxidase. After a final wash with PBS, HRP-bound sites were developed with the chromogenic reagent 3,3′ diaminobenzidine (DAB). All sections were processed during the same session and had the same development time. Sections were lightly stained with haematoxylin, dehydrated and mounted. Negative controls were performed as above, replacing the primary antibody with PBS.

2.2.2. Double immunofluorescence staining of ACTH and ERα in pituitary gland

ACTH and ERα colocalisation was evaluated. The pituitary gland from each animal was fixed, embedded in paraffin, sectioned and rehydrated as described previously for "*Immunostaining of ACTH in pituitary gland*". REα antigen retrieval was performed. With this purpose, sections were placed in citrate buffer (0.01 M, pH = 6.0) and heated in the microwave (900 W) for 10 min. Sections were allowed to cool at room temperature and then washed with PBS. Non-specific binding sites were blocked with normal goat serum ("Immunoperoxidase secondary detection system," DAB 150, Millipore, CA, USA) for 10 min. Sections were incubated overnight at room temperature in a humidified chamber with the anti-ERα primary antibody (mouse monoclonal, sc-787, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:25 in PBS. The sections were washed with PBS and incubated for 1 h with the anti-mouse biotinylated antibody (E0354, DAKO) diluted 1:125 in PBS, which was followed by a 1 h incubation with streptavidin-conjugated FITC (SA 100-02, Molecular Probes, Invitrogen) diluted 1:100 in PBS. After washing with PBS, the sections were incubated overnight in a humidified chamber at room temperature with the anti-ACTH primary antibody (rabbit-anti human ACTH, NIDDK; raised against 1–24 amino acids) (it was chosen in order to use an antibody raised in a different species than the ERα antibody) diluted 1:100 in PBS. The sections were washed with PBS and incubated for 1 h with anti-rabbit rhodamine antibody (AP132R; Chemicon International) diluted 1:100 in PBS. Finally, the sections were mounted in PBS-glycerol (1:1). Negative controls were performed as above, replacing the primary antibody with PBS.

2.2.3. Immunostaining for ERα and MC2R in adrenal glands

The adrenal glands of all dogs were fixed, embedded in paraffin, sectioned and rehydrated using the protocol previously described for the immunostaining in pituitary glands. Antigen retrieval was performed on sections used for ERα immunostaining as described for the pituitaries. All of the sections were then incubated with 3% H_20_2 in distilled water for 20 min before being washed with PBS. Non-specific binding sites were blocked with normal horse serum for 30 min (Vectastain ABC-Kit, Elite, PK-6102, Burlingame, CA, USA). Sections were incubated in a humidified chamber for 2 h with either a 1:100 PBS:anti-MC2R primary antibody dilution (rabbit polyclonal; sc-13107, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or with a 1:25 dilution of the anti-ERα primary antibody (mouse monoclonal, sc-787, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS (Ithurralde et al., 2013). After being washed with PBS, sections were incubated for 1 h with a 1:200 dilution of the biotinylated anti-rabbit antibody (BA-1000, Vector, Burlingame, CA, USA) or the biotinylated anti-mouse antibody (BA-200, Vector, Burlingame, CA, USA) as appropriate for each section based on the previously applied primary antibody. Sections were washed with PBS before use of the "Vectastain Elite ABC Kit" detection system (Vector, Burlingame, CA, USA). The following steps were completed as described for sections of the pituitary gland. In all cases, negative controls were conducted via replacement of the primary antibody with PBS.

2.3. Image analysis

The images of the single immunohistochemistry performed in pituitaries and adrenal glands were captured with a digital Leica DCC-380× camera attached to a trinocular microscope (Leica, DM4000B Led) at 1000× magnification using Las Leica Inc. Software and analysed using Qwin Plus Leica Inc. Corp software. The pituitary gland samples were analysed with images from 40 non-consecutive fields per section, randomly selected, to determine the number of immunostained cells/total cells per field (proportion of corticotrophs per field) (field = 12,262 μ^2). Corticotrophs were characterised using the following parameters: cellular area, nuclear and cytoplasmic area and optical density. With this goal, 100 immunostained cells per section were analysed.

ERα expression in the adrenal zona fasciculata was evaluated using the number of immunostained cells/total cells per field in images from 40 non-consecutive fields, randomly selected (the glomerulosa and zona reticularis were excluded from this study) (field = 12,262 μ^2). MC2R expression was evaluated based the optical density of 100 immunopositive cells per section.

In the analysis of both the pituitary and adrenal glands, the optical density analysis was performed using images transformed to 8 bit (grey scale). The optical density was calibrated using a step tablet specifically calibrated for this purpose. The calibration curve was extended from 0 to 2.6 (optical density values). Using this system, as the immunostaining intensity increased, so did the optical density (Oberholzer et al., 1996; Optical density calibration).

The images of the double immunofluorescence staining were analysed using a confocal laser microscope (Olympus FV-30 attached to an Olympus Bx-61 microscope).

2.4. Statistical analysis

The data obtained (proportion of immunostained cells per field, cellular area, nuclear area, cytoplasmic area, and optical density) was analysed using a two-way analysis of variance (ANOVA) followed by a Bonferroni test that considered the sex of the animal, season and interaction between these two factors. The individuals (dogs) were set as random effect (Graph Pad 5, USA). The values were expressed as the mean ± SD. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Pituitary gland

3.1.1. Immunopositive cells/total cells per field (proportion of corticotrophs)

As no interaction was found between sex and season in this study, single effect was analysed. Sex had an effect on the proportion of positive cells per field ($p < 0.0001$), but the season did not. The number of positive cells/total cells per field was greater in females than males in both seasons ($p < 0.01$) (Fig. 1). In the summer, the proportion of corticotrophs per field was 0.32 ± 0.02 in females and 0.22 ± 0.04 in males. In the winter, the proportion of corticotrophs per field was 0.34 ± 0.03 in females and 0.21 ± 0.01 in males.

3.1.2. Cellular, nuclear and cytoplasmic area

As no interaction was found between sex and season in this study, single effect was analysed. Sex affected the cellular area ($p < 0.0001$) but season did not. Total cellular area was greater in females than in males (84.3 μ^2 ± 0.3 μ^2 vs. 73.7 μ^2 ± 0.3 μ^2, respectively) ($p < 0.001$). The nuclear area was not affected by sex or season. However, cytoplasmic area was affected by sex ($p < 0.0001$), although not by season. Cytoplasmic area was greater in females than in males ($p < 0.001$) in both seasons. The cytoplasmic area was 68.1 μ^2 ± 0.25 μ^2 in females and 57.5 μ^2 ± 0.3 μ^2 in males.

3.1.3. Optical density

The corticotroph optical density was affected by both sex ($p < 0001$) and season ($p < 0.0001$), but there was no interaction between them. The optical density was greater in females than in males in both seasons ($p < 0.001$). Similarly, the optical density of these cells was greater in the winter in both sexes compared with the summer ($p < 0.001$) (Fig. 2).

3.1.4. ERα expression in corticotrophs

The ERα was present in the nucleus of the corticotrophs in both sexes. The relatively few corticotrophs that expressed ERα were distributed throughout several regions of the anterior pituitary. Similarly, a large number of ERα-immunopositive nuclei were observed in the proximity of these cells (Fig. 3). No quantitative analysis of ERα expression in corticotrophs was conducted because of low expression.

3.2. Adrenal cortex

3.2.1. ERα expression

ERα was present in the zona fasciculata of all of the adrenal glands studied (Fig. 4).

The proportion of immunostained cells per field was affected by both sex ($p < 0.0001$) and season ($p < 0.0001$), without interaction between these factors. ERα was expressed at higher levels (greater number of immunostained cells/total cells per field) in females than in males in both seasons ($p < 0.001$) (Fig. 5). The proportion of immunostained cells was greater in winter than summer for both sexes ($p < 0.001$) (Table 1).

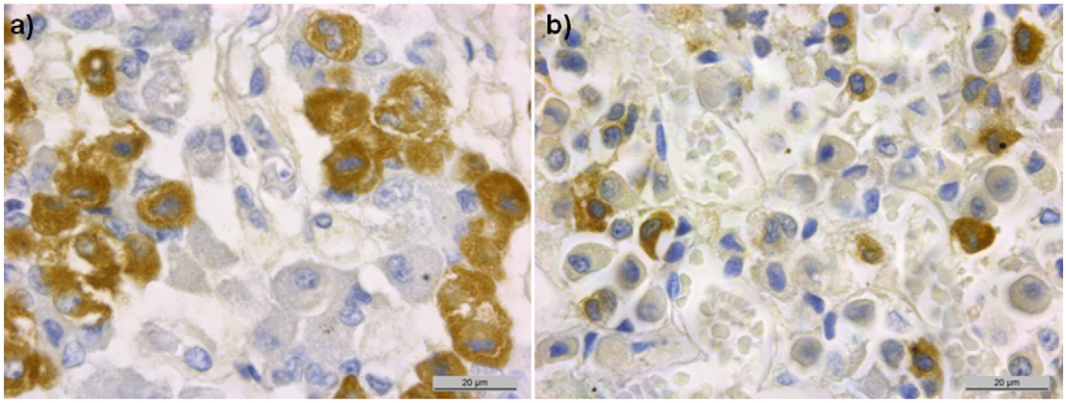

Fig. 1. Immunostaining for ACTH in the pituitary gland of a female (a) and male (b) dog in winter. Corticotrophs are marked by brown-stained cytoplasms. The scale bar indicates 20 µm.

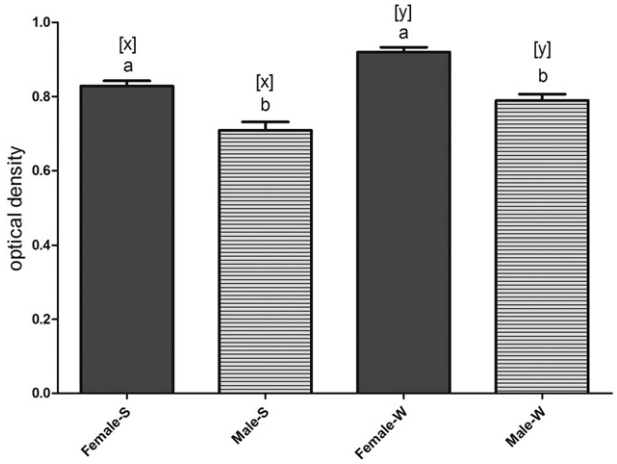

Fig. 2. The optical density of corticotrophs in males and females in different seasons. a vs. b indicate significant sex differences within season ($p < 0.001$). [X] vs. [y] indicate significant differences between seasons ($p < 0.001$). Female-S: females in summer, Male-S: males in summer, Female-W: females in winter, Male-W: males in winter.

3.2.2. MC2R expression

Optical density was affected by sex ($p < 0.0001$) and season ($p < 0.0001$), without interaction between these factors. In both seasons, cells in the zona fasciculata had a greater optical density in females than in males ($p < 0.001$) (Figs. 4, 6). Similarly, the optical density was greater in winter than summer for both sexes ($p < 0.001$) (Table 1).

4. Discussion

4.1. Sex-based morphological analysis in dogs

Sexual dimorphism was observed in this study, both in the morphological characteristics of corticotrophs and receptor expression in the zona fasciculata of the adrenal gland in dogs.

It is important to consider that this work has a logical limitation since the studied dogs were not experimental animals, but were patients of the Veterinary Hospital of the University of Buenos Aires that were euthanized for humanitarian reasons (they were 14 females and 13 male dogs which were not breed matched, although they were matched by weight).

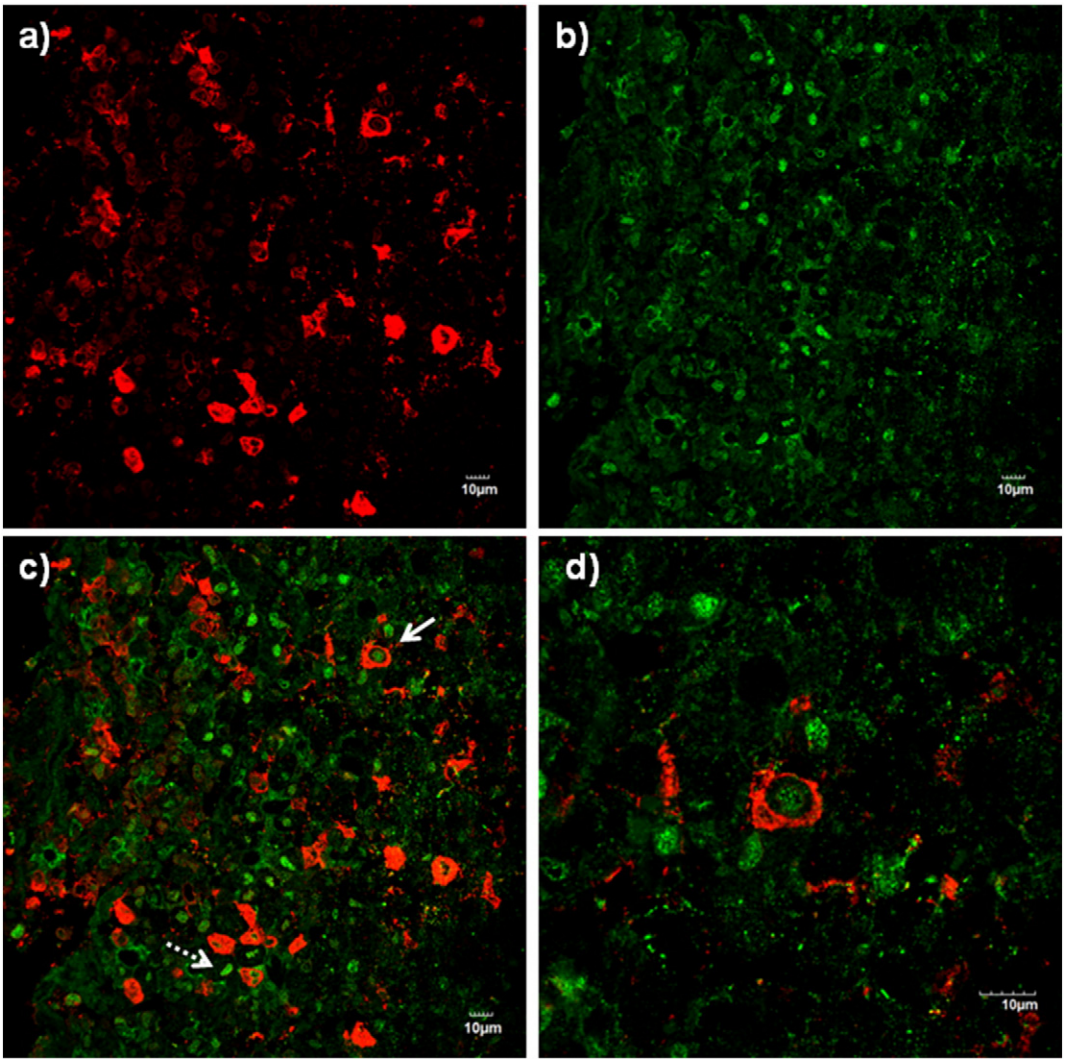

Fig. 3. Co-localisation of ERα in corticotrophs in dogs, detected via double immunofluorescence. Images were obtained using a confocal microscope. (a) Immunostaining of ACTH in corticotrophs (red), (b) immunostaining of nuclei expressing ERα (green), (c) co-localisation of both antigens (nuclei in green and cytoplasms in red), and (d) higher magnification of a cell positive for both ERα and ACTH. The solid arrow indicates co-localisation. The dotted arrow indicates a non-corticotroph cell that is positive for ERα. Scale bar indicates 10 µm.

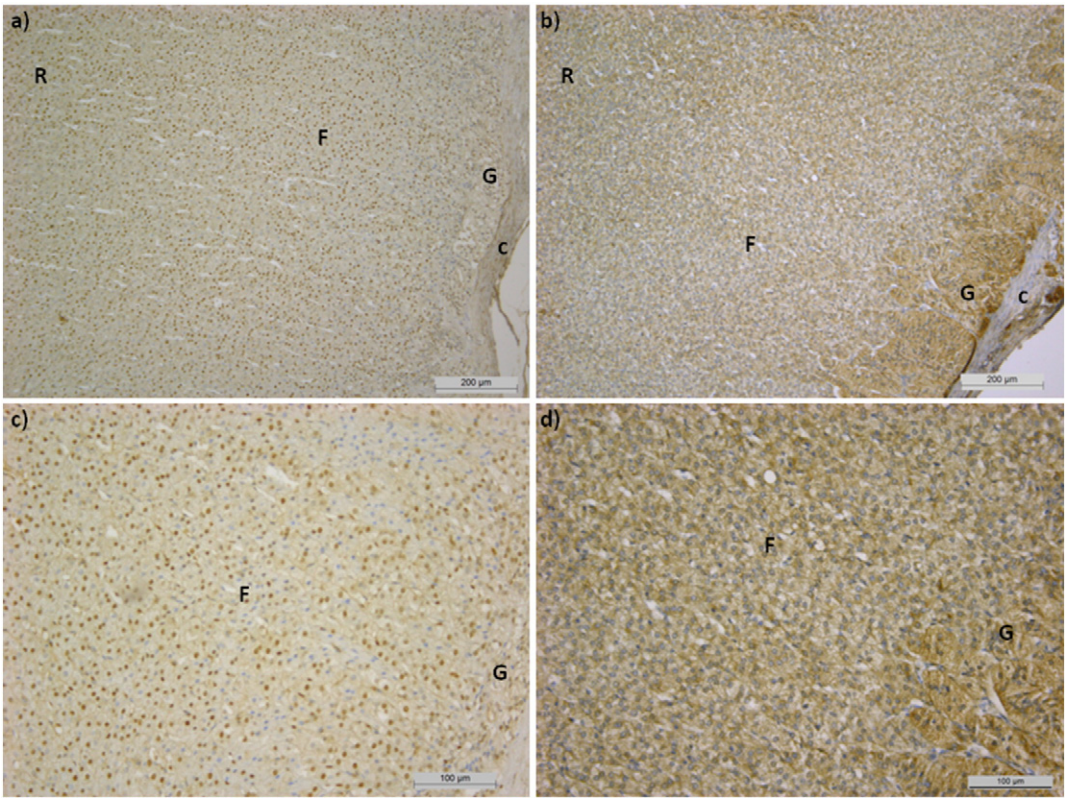

Fig. 4. Immunostaining for ERα (a, c) and for MC2R (b, d) in the adrenal cortex of a female dog. Brown nuclei (a, c) and brown cytoplasm (b, d) indicate positive staining. The scale bar indicates 200 µm (a, b) or 100 µm (c, d). [C]: capsule, [G]: zona glomerulosa, [F]:zona fasciculata, [R]:zona reticularis.

The ethics committee did not allow euthanizing healthy dogs, however we consider that the studied animals provide a good model to approach to HPA axis's morphological characteristics.

In both seasons studied, female dogs had a greater proportion of corticotrophs, greater cell size (related to a greater cytoplasm area) and greater optical density (greater immunostaining intensity).These differences suggest that there could be an impact of sexual hormones on these cells. The female's corticotroph morphological characteristics could render their HPA axis more sensitive to stress, as it has been described in rats (Goel et al., 2014). Vidal et al. (1995) and Filippa and Mohamed (2006) reported sex-linked morphological differences in the American vison and vizcachas. Both studies suggest that these observations were because of the effects of sexual steroids on this group of pituitary cells. Several authors have described the stimulatory role of oestradiol on the HPA axis, specifically on the pituitary gland and hypothalamus and/or hippocampus (Turner, 1990; Burgess and Handa, 1993; Panagiotakopoulos and Neigh, 2014; Handa and Weiser, 2014). Similarly, Weiser and Handa (2009) showed that ERα is present in the paraventricular nucleus in rats, explaining that oestradiol may inhibit negative feedback from the glucocorticoids at a central level. ERα was also identified in the corticotrophs of rats (Mitchner et al., 1998) where oestradiol could also exert a regulatory effect on the HPA axis. Coinciding with these publications, this study found, for the first time, ERα expression in the nucleus of corticotrophs in dogs suggesting that oestradiol might exert an effect on these cells through binding with ERα (Levin, 2005; Handa and Weiser, 2014). Furthermore, ERα was present in cells adjacent to corticotrophs. Oestradiol may directly affect these cells and/or induce them to exert a paracrine effect on corticotrophs (Denef, 2008); this hypothesis must be addressed in future studies.

The ERα was identified not only in the pituitary gland but also in cells of the zona fasciculata of the adrenal gland in dogs, and to our

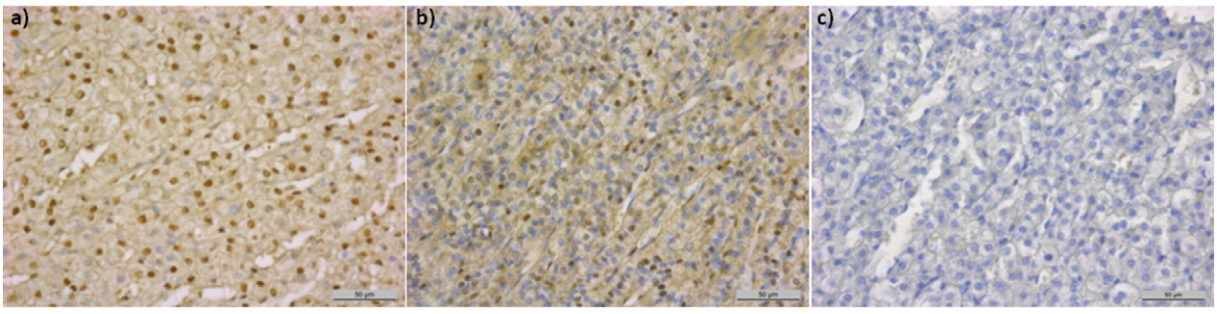

Fig. 5. Immunostaining for ERα in the adrenal cortex (zona fasciculata) a female (a) and male (b) dog. Brown nuclei indicate positive staining while blue nuclei (c) are negative (haematoxylin). The scale bar indicates 50 µm.

Table 1
Expression of ERα and MC2R in the adrenal zona fasciculata of dogs in different seasons.

Receptor	Female		Male		*p*-Value	
	Summer	Winter	Summer	Winter	Sex	Season
ERα (IP/T cells)	0.51 ± 0.02	0.56 ± 0.02	0.13 ± 0.01	0.17 ± 0.01	<0.001	<0.001
MC2R (O.D.)	0.63 ± 0.02	0.75 ± 0.05	0.54 ± 0.02	0.63 ± 0.02	<0.001	<0.001

IP/T cells: immunopositive cells/total cells per field.
O.D.: optical density.
Values are expressed as the mean and standard deviation.

knowledge this is the first time that this finding has been reported in this species. This receptor in the adrenal cortex suggests an additional active site for oestradiol's influence on the HPA axis. The presence of ERα in the adrenal glands has been described in other species such as rats and sheep; and it has been suggested that by binding to it, oestradiol could modulate adrenal steroidogenesis (Lo et al., 2000; van Lier et al., 2003). Furthermore, ERα expression in the zona fasciculata was greater in female dogs than in male dogs. Therefore oestradiol has more available binding sites in females. The MC2R expression was also greater in females than in males. In rats it has been reported that females show higher glandular sensitivity to ACTH and that oestradiol could increase MC2R expression (Lo et al., 2000; Mitsushima et al., 2003; Figueiredo et al., 2007). Although performing molecular studies would provide information about RNA expression or a Western blot analysis would provide a more precise quantification of protein expression, in the present study we used immunohistochemistry to assess receptor's expression in a certain cell type (corticotrophs or zona fasciculata cells). We aim to perform further studies analysing RNA expression and its relation to protein expression.

In the present study, sex- linked differences in the HPA axis were observed at a morphological level in dogs. However, sex- linked functional differences in the HPA axis have also been reported in other species: basal glucocorticoid secretion and secretion in response to stress are greater in females than males in rats and sheep (Handa and Weiser, 2014; van Lier et al., 2014). Similarly, in rats CRH and ACTH secretion is greater in females than in males (Handa et al., 1994; Mc Cormick et al., 1998; Viau et al., 2005; Panagiotakopoulos and Neigh, 2014). Pessina et al. (2009) observed a greater response to ACTH stimulation and dexamethasone inhibition in female dogs than in males. Our group has also found differences in ACTH and cortisol concentrations in the plasma in different stages of the oestrous cycle of the dog (Gallelli et al., 2015). Consequently, through binding to its receptor, oestradiol could determine the histological differences observed in this study and maybe modulate the synthesis and/or secretion of ACTH and cortisol, thereby generating functional differences between the sexes (Rhodes and Rubin, 1999). This hypothesis remains to be evaluated.

4.2. Seasonal morphological analysis

This study did not observe seasonal differences in corticotroph proportion or size, although differences in corticotroph optical density were observed. Optical density was greater in both sexes during winter, which could be related to a greater ACTH expression in corticotrophs during the same period. The greater ACTH expression would provide a hormone reserve for the HPA axis that could be liberated and rapidly stimulate the secretion of glucocorticoids in periods of unfavourable climactic conditions or metabolic changes as it has been proposed in horses (Donaldson et al., 2005; Cordero et al., 2012).Seasonal variations were also observed in the morphology and staining intensity in the corticotrophs of frogs (Pramoda and Saidapur, 1991) and in histomorphological characteristics of these cells in rodents and vizcachas, suggesting that corticotrophs would respond to the effects of melatonin on the pituitary gland (Hira et al., 2001; Filippa and Mohamed, 2006). Seasonal variations were also observed at the level of the adrenal gland, with MC2R expression greater in the winter. This increase in expression could augment the capacity of the adrenal gland to respond to ACTH stimulation. A study of the sand rat (*Psammomys obesus*) reported increased adrenal sensitivity to ACTH during winter, which in turn allowed for an increase in the secretion of cortisol and maintenance of cortisol levels until spring (Amirat and Brudieux, 1993). The ERα expression in adrenal glands also increased in dogs during the winter. This finding has not been previously reported, and the associated mechanisms are unknown.

Our group has observed that ACTH and cortisol plasma concentrations during the oestrus cycle of female dogs are greater in winter (Gallelli et al., 2015). Thus we hypothesise that there could be a relationship between HPA axis function and seasonal differences in corticotroph optical density and receptor expression in the adrenal gland. Similarly, seasonal variations in HPA axis-associated hormones

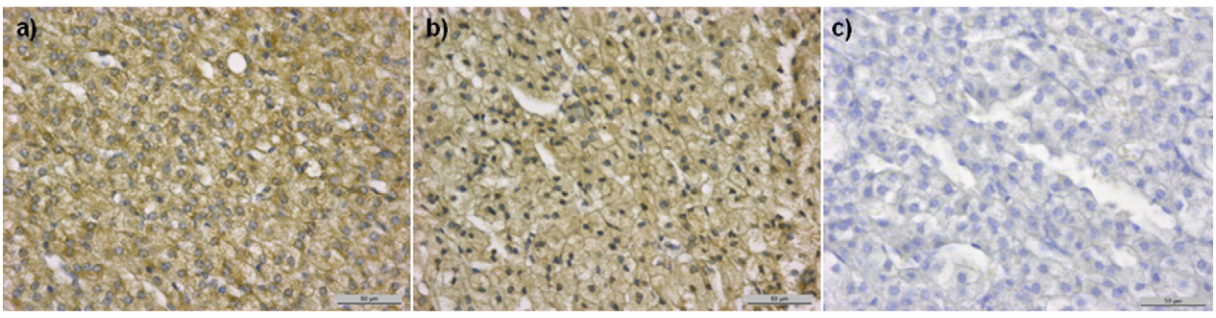

Fig. 6. Immunostaining for MC2R in the adrenal cortex (zona fasciculata) of a female (a) and male (b) dog, and negative control (c). Brown cytoplasms indicate positive immunostaining. The difference in intensity between both samples can be seen. The scale bar indicates 50 μm.

have been reported in other species (Romero, 2002; Donaldson et al., 2005; Cordero et al., 2012).

5. Conclusions

Sex-based differences were found in the morphological characteristics of corticotrophs and receptor expression in the adrenal zona fasciculata. The presence of ERα in corticotrophs and the adrenal cortex may indicate a site of oestradiol action through which it can exert influence on the HPA axis. Furthermore, seasonal differences were found in the expression of receptors in the adrenal gland as well as in the optical density of corticotrophs, which may be related to metabolic or climactic adaptations. The observed differences between sexes and seasons provide new information about HPA axis' morphological characteristics and maybe could explain its functional dimorphism. This hypothesis remains to be studied.

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