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Effects of castration on androgen receptors and gonadotropins in the pituitary of adult male viscachas

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Abstract. The aims of the present study were to determine whether castration results in quantitative immunohistochemical changes in androgen receptors (AR), LH-immunoreactive (IR) cells and FSH-IR cells, and to analyse the colocalisation of AR and gonadotropins in the pituitary pars distalis (PD) of viscachas. Pituitaries were processed for light and electron microscopy. AR-IR, LH-IR and FSH-IR cells were detected by immunohistochemistry. In morphometric studies, the percentage of AR-IR, LH-IR, FSH-IR, LH-IR/AR-IR and FSH-IR/AR-IR cells was determined. In intact viscachas, AR were distributed throughout the PD; they were numerous at the caudal end, with intense immunostaining. LH-IR cells and FSH-IR cells were found mainly in the ventral region and at the rostral end of the PD. Approximately 45%–66% of LH-IR cells and 49%–57% of FSH-IR cells expressed AR in the different zones of the PD. In castrated viscachas, there was a significant decrease in the percentage of AR-IR, LH-IR, FSH-IR, and FSH-IR/AR-IR cells. Some pituitary cells from castrated viscachas also exhibited ultrastructural changes. These results provide morphological evidence that gonadal androgens are directly related to the immunolabelling of AR, LH and FSH. Moreover, the colocalisation of AR and FSH is most affected by castration, suggesting the existence of a subpopulation of gonadotrophs with different regulatory mechanisms for hormonal synthesis, storage and secretion.

Additional keyword: immunocytochemistry.

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Introduction

Sex steroid hormones play important roles in the control of reproductive behaviour and metabolic function in males. Androgens are produced primarily by the Leydig cells of the testis. Androgens act via androgen receptors (AR) and can affect the function of a variety of cells in several organs (Handa *et al.* 1987; Beato *et al.* 1995). In the hypothalamo–pituitary–gonadal axis, androgens negatively regulate gonadotropin secretion and thus reproductive function (Kalra and Kalra 1983; Gharib *et al.* 1990). Okada *et al.* (2003) have reported that androgens not only decrease gonadotropin-releasing hormone (GnRH) in male rats, but also suppress LH synthesis and secretion via a direct effect on the pituitary. Spady *et al.* (2004) have demonstrated, using mouse gonadotrophs, that androgens act directly on the *FSHβ* gene, stimulating expression by different mechanisms.

Pituitary AR have been studied using immunohistochemistry in several species (Sar *et al.* 1990; Iqbal *et al.* 1995; Lindzey *et al.* 1998; Abdelgadir *et al.* 1999; Pelletier *et al.* 2000). AR have been found in either occupied (nuclear receptors; ARn) or unoccupied (cytoplasmic receptors; ARc) conditions. The expression of these receptors has been observed mainly in gonadotrophs (Kimura *et al.* 1993; Yuan *et al.* 2007; Scheithauer *et al.* 2008) and, to a lesser extent, in somatotrophs (Wehrenberg and Giustina 1992; Kimura *et al.* 1993) and lactotrophs (Stefaneanu 1997; Yuan *et al.* 2007). In many androgen-dependent target tissues, castration is associated with a decline in ARn and an increase in ARc (Connolly *et al.* 1991; Iqbal *et al.* 1995). Moreover, increased production of gonadotropins (Gharib *et al.* 1987; Childs *et al.* 1990) and ultrastructural changes in gonadotrophs (Yoshimura and Harumiya 1965; Ibrahim *et al.* 1986; Cónsole *et al.* 2001) have been observed in the pituitary of castrated animals.

The viscacha (*Lagostomus maximus maximus*) is a nocturnal rodent with seasonal reproductive patterns. In their natural habitat, adult male viscachas exhibit a reproductive cycle synchronised by the environmental photoperiod through the pineal gland and its main hormone melatonin (Domínguez *et al.* 1987; Pelzer *et al.* 1999). The reproductive cycle of the viscacha is characterised by three periods: (1) gonadal regression; (2) gonadal recovery; and (3) the reproductive period. During the gonadal regression period, which occurs during the short days of winter, analysis of different morphometric parameters has revealed reduced activity of hormone-producing cell types in the pars distalis (PD) of adult male viscachas (Filippa *et al.* 2005; Filippa and Mohamed 2006*a*, 2006*b*, 2008, 2010). In addition, a significant decrease in morphometric parameters of somatotrophs, thyrotrophs and lactotrophs was observed in the PD of the pituitary of castrated viscachas, indicating that androgens affect the activity of these cell types (Filippa and Mohamed 2006*b*, 2008, 2010). No information is available regarding the expression of AR and their colocalisation with gonadotropins (LH and FSH) in the pituitary PD of castrated adult male viscachas. The aims of the present study were to investigate the effects of castration on the immunolabelling of AR, LH-immunoreactive (IR) cells and FSH-IR cells in the pituitary PD of adult male viscachas, and to analyse the colocalisation of AR and gonadotropins to determine whether castration has any effects. Moreover, ultrastructural changes in the PD of viscachas were evaluated.

Materials and methods

Animals

The viscachas were captured in their habitat near San Luis, Argentina (33°20′S, altitude 760 m) using traps placed in their burrows.

Sixteen adult male viscachas captured during the reproductive period (summer and early autumn) were used. The animals were divided into two groups: (1) a group of surgically castrated viscachas ($n = 8$); and (2) a control group of intact viscachas ($n = 8$). The viscachas were kept individually in boxes for 6 weeks and maintained under a 14 : 10 h light–dark cycle, with free access to food and water, at 20° ± 2°C, as used in studies (Filippa and Mohamed 2006*b*). After 6 weeks, the viscachas were anaesthetised with an intraperitoneal injection of 0.3 mL kg^{-1} of a 10 : 1 (w/v) mixture of ketamine (ketamine hydrochloride; Holliday Scott SA, Buenos Aires, Argentina) : xylazine (xylazine hydrochloride; Richmond Laboratories, Veterinary Division SA, Buenos Aires, Argentina). The viscachas were then killed by intracardiac injection of 2.5 mL kg^{-1} Euthanyle (sodium pentobarbitone, sodium diphenylhydantoin; Brouwer SA, Buenos Aires, Argentina). The reproductive condition of the viscachas in the control group was carefully assessed using light microscopy observations of the testes. All control male viscachas exhibited morphological characteristics of gonadal activity according to Muñoz *et al.* (1998). After the viscachas had been killed, their brain was exposed rapidly and the pituitary processed for light and electron microscopy. Four pituitary glands from castrated viscachas and four from intact animals were used for light microscopy. The pituitaries were sectioned sagittally and the hemipituitaries fixed in Bouin's fixative, embedded in paraffin and then sectioned sagittally (5 µm), as described previously (Filippa and Mohamed 2006*b*, 2010). For transmission electron microscopy, pituitaries (four each from castrated and intact viscachas) were fixed in Karnovsky's fixative (Karnovsky 1965), post-fixed in 1% osmium tetroxide for 2 h at 4°C, washed in phosphate buffer (pH 7.2–7.4), dehydrated in acetone and embedded in Spurr plastic resin. Consecutively sections (1 µm) were stained with 1% toluidine blue for morphological evaluation. For electron microscopy, ultrathin sections were cut with a Porter-Blum ultramicrotome (Ivan Sorvall, Norwalk, CT, USA), and then contrasted with lead citrate and uranyl acetate (Millonig 1961). Ultrastructural characteristics were investigated in detail under a Siemens Elmiscop I electron microscope (Siemens Co., Berlin, Germany).

The experimental design was approved by the local Ethics Committee of Universidad Nacional de San Luis, and was in agreement with the National Institutes of Health (NIH) guidelines for the use of experimental animals. Moreover, the Biodiversity Control Area of the San Luis Ministry of the Environment (Argentina) approved a study protocol for conducting scientific research within the territory of this province (Resolution No. 03 PRN-2011).

Single immunohistochemistry for AR, LH-IR cells and FSH-IR cells in the pituitary

Tissue sections were stained using the streptavidin–biotin–peroxidase complex method at 20°C. Sections were first deparaffinised with xylene, hydrated through decreasing concentrations of ethanol and rinsed with distilled water and phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Antigen retrieval was performed by microwaving the sections for 6 min (2 × 3 min) at full power in a 900 W microwave oven in sodium citrate buffer (0.01 M, pH 6.0). Endogenous peroxidase activity was inhibited with 3% H_2O_2 in water for 20 min. Non-specific binding sites for immunoglobulins were blocked by incubation of sections for 20 min with normal serum diluted in PBS containing 1% bovine serum albumin, 0.09% sodium azide and 0.1% Tween-20. Sections were incubated with the primary antibody as follows: for 6 h in a humidified chamber at 20°C with rabbit polyclonal anti-human (h) AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA); or for 12 h in a humidified chamber at 4°C with monoclonal anti-hLHβ (Clone 3 LH 5B6 YH4; BioGenex, San Ramón, CA, USA) or monoclonal anti-hFSHβ (Clone 83/12/2A82C7; BioGenex). After rinsing with PBS for 10 min, immunohistochemical visualisation was performed using the Super Sensitive Ready-to-Use Immunostaining Kit (BioGenex), as follows: sections were incubated for 30 min with diluted biotinylated anti-IgG and, after washing in PBS, were incubated for 30 min with horseradish peroxidase-conjugated streptavidin and finally washed in PBS. The reaction sites were visualised using freshly prepared solutions of 100 µL of 3,3′-diaminobenzidine tetrahydrochloride chromogen in 2.5 mL PBS and 50 µL H_2O_2 substrate solution. Sections were counterstained with Harris' haematoxylin for 10 s, dehydrated and mounted. The cells identified by single immunohistochemistry were as follows: (1) AR-IR cells (cells containing AR); (2) LH-IR cells (cells containing LH); and (3) FSH-IR cells (cells containing FSH).

In all cases, two experiments for controlling the specificity of the primary antibody were performed: (1) omission of the primary antibody, and (2) adsorption of the primary antibody with a homologous antigen. No positive structures or cells were found in these sections. Rat pituitary and prostate were used as positive controls (Fig. 1).

Double immunohistochemistry for AR and LH or FSH in the pituitary

Double immunohistochemistry was performed with the objective of examining the expression of AR in LH-IR and FSH-IR cells in the pituitary PD. Diaminobenzidine (DAB) and New Fuchsin (BioGenex, San Ramon, CA, USA) were selected as

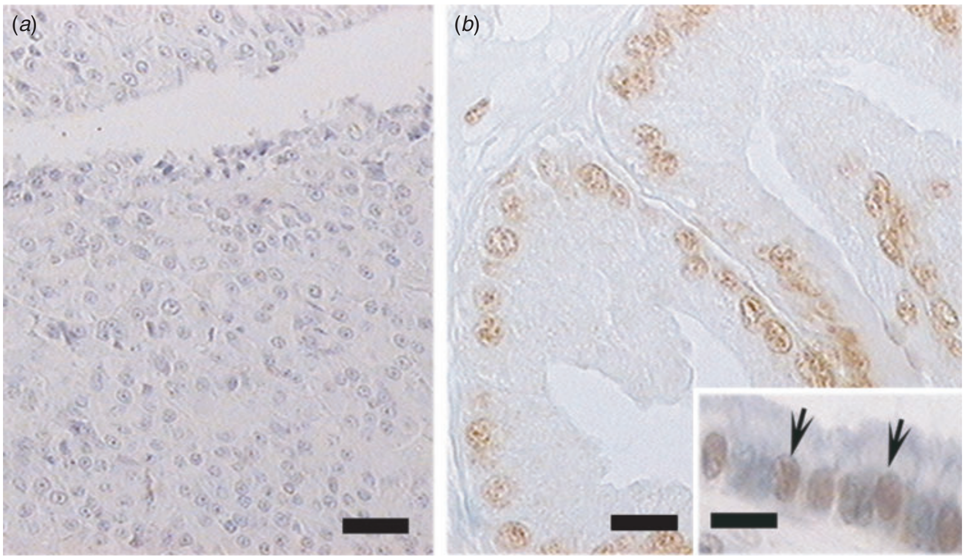

Fig. 1. (*a*) Viscacha pituitary: negative immunohistochemical control of counterstaining with haematoxylin. Scale bar = 50 μm. (*b*) Rat prostate: positive control of immunoperoxidase staining with polyclonal anti-human (h) androgen receptor (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) without haematoxylin staining. Inset: immunolabelled nuclei of epithelial cells (arrows) counterstained with haematoxylin. Scale bars = 25 μm.

chromogens to visualise the antigens because this combination is known to give good contrast (Acosta *et al.* 2010). Sections used for the single labelling of AR were stained for double-labelling of AR and LH or FSH. Prior to LH or FSH staining, sections were stained in the same manner as for single labelling of AR, using DAB as the chromogen. Then, slides were washed (3 × 10 min) in 0.1 M glycine-HCl buffer (pH 2–2.2) at 20°C for 70 min, then washed in PBS and finally incubated with the second primary antibody for 12 h in a humidified chamber at 4°C (against LH or FSH). The slides were then washed (3 × 10 min) in PBS and the sections incubated for 30 min with biotinylated anti-IgG, washed (3 × 5 min) in PBS and incubated for 30 min with alkaline phosphatase-conjugated streptavidin. Finally, sections were washed in PBS for 10 min and the reaction sites were revealed using 100 μL New Fuchsin from the New Fuchsin Chromogen Kit (catalogue no. HK 183-5K; BioGenex), resulting in a fuchsia-coloured precipitate. The sections were then counterstained with Harris' haematoxylin for 10 s, washed for 2 min under running water and mounted with permanent aqueous mounting medium (SuperMount; BioGenex). Labelling was assessed using under a light microscope (BX-40; Olympus Optical, Tokyo, Japan).

The cells identified by double immunohistochemistry were LH-IR/AR-IR cells (cells that contained LH and nuclear AR) and FSH-IR/AR-IR cells (cells that contained FSH and nuclear AR).

Single and double immunohistochemical procedures were similar to those reported previously (Filippa *et al.* 2005, 2012; Acosta *et al.* 2010).

Morphometric analysis

A computer-assisted image analysis system was used for morphometric analysis, as reported previously (Filippa *et al.* 2012). Briefly, the image was displayed on a colour monitor, a standard area of 18141.82 μm^2 (reference area) was defined on the monitor, and distance calibration was performed using a slide with a micrometric scale for microscopy (Reichert, Vienna, Austria). Eight pituitaries were analysed, four from each of the control and castrated groups. The morphometric study was performed as follows: four regularly spaced serial tissue sections (100 μm each) from a pituitary were used and microscopic fields were examined under a ×40 objective. In each section, 25 microscopic fields were randomly selected throughout the PD (five from each region or end of the PD: ventral, medial and dorsal regions, rostral and caudal ends). The following morphometric parameters listed below were evaluated.

1. In each image (~250–280 cells), the percentage of single immunoreactive cells in the PD (i.e. percentage of ARn-IR, ARc-IR, LH-IR and FSH-IR cells) was determined using the formula $A/(A+B) \times 100$, where A is the number of single immunoreactive cells and B is the number of nuclei in immunonegative cells.
2. In each image, the percentage of LH-IR cells expressing nuclear AR (i.e. LH-IR/AR-IR cells) was obtained using the formula $A/(A+B) \times 100$, where A is the number of LH-IR/AR-IR cells and B is the number of immunolabelled LH-IR cells.
3. In each image, the percentage of FSH-IR cells expressing nuclear AR (i.e. FSH-IR/AR-IR cells) was obtained using the formula $A/(A+B) \times 100$, where A is the number of FSH-IR/AR-IR cells and B is the number of immunolabelled FSH-IR cells.

Statistical analysis

Results are expressed as the mean ± s.e.m. Differences between the two groups (control vs castrated) were evaluated using

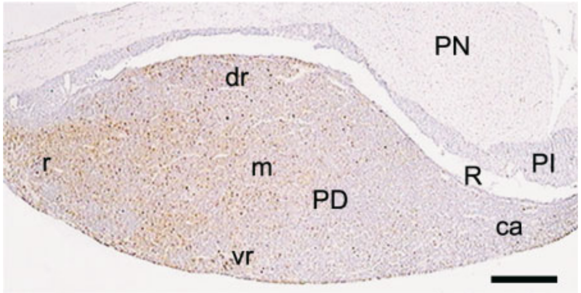

Fig. 2. Sagittal section of a pituitary from an adult male viscacha showing the different regions. PD, pars distalis; PI, pars intermedia; PN, pars nervosa; R, Rathke's pouch; ca, caudal end; r, rostral end; vr, ventral region; m, medial region; dr, dorsal region. (Immunohistochemical technique with anti-FSH antibody.) Scale bar = 500 µm.

Student's *t*-test. The significance of differences between groups in the percentages in different regions and ends of the PD were evaluated by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test. $P < 0.05$ was considered significant.

Results

There are different zones in the pituitary PD: the ventral (anterior), medial and dorsal (posterior, close to Rathke's pouch) regions, and the rostral (or cephalic, superior, connected to the pars tuberalis) and caudal (inferior) ends (Fig. 2). Cells were counted in each of the different zones of the pituitary PD parenchyma of adult male viscachas 6 weeks after castration.

AR-IR cells were observed throughout the parenchyma of the PD. Immunohistochemical results revealed that AR-IR was detected mainly in the nuclei of PD cells (ARn-IR), but cytoplasmic immunolabelling (ARc-IR) was also observed in some cells. The nuclei AR-IR cells were oval or spherical in shape. Nuclear immunolabelling was less intense in cells localised in the medial region and more intense at the caudal end. In the control group, the percentage of ARn-IR cells was higher at the caudal end than in the other PD regions. In castrated viscachas, the percentage of ARn-IR cells was higher at the caudal end compared with the ventral and medial regions, as well as the rostral end. In addition, in all PD zones of castrated viscachas, the percentage of ARn-IR and ARc-IR cells decreased significantly compared with the control group (Table 1; Fig. 3*a–d*).

LH-IR and FSH-IR cells were distributed in all PD zones. They were numerous in the ventral region and less numerous at the caudal end. These cells were arranged in cellular cords, grouped or isolated, covering blood vessels and near follicular structures. Gonadotrophs were oval or spherical in shape, with an eccentric nucleus. In the control group, the cytoplasmic immunolabelling pattern was homogeneous. The percentage of LH-IR and FSH-IR cells in the ventral region was significantly higher that at the caudal end (Fig. 4). In castrated viscachas, some LH-IR and FSH-IR cells exhibited hypertrophic-like morphology with a heterogeneous cytoplasmic immunolabelling pattern. These IR cells were found mainly in the ventral and medial regions, and their arrangement in the parenchyma was similar to that in the control group. The percentage of LH-IR cells at the

Table 1. Percentage of cytoplasmic and nuclear androgen receptor-immunoreactive (AR-IR) cells

Values are the mean ± s.e.m. ($n = 4$). *$P < 0.05$, **$P < 0.01$ compared with control (Student's *t*-test). $^{\dagger}P < 0.01$ compared with the caudal end (ANOVA followed by the Tukey–Kramer test)

PD zones	%AR-IR cells			
	Cytoplasmic		Nuclear	
	Control	Castrated	Control	Castrated
Ventral region	2.78 ± 0.61	0.24 ± 0.04*	14.48 ± 0.99†	6.93 ± 1.49**†
Dorsal region	1.96 ± 0.51	0.53 ± 0.18*	17.02 ± 0.53†	10.23 ± 2.08*
Medial region	1.44 ± 0.06	0.28 ± 0.03**	11.93 ± 0.95†	7.89 ± 1.08*†
Rostral end	2.35 ± 0.57	0.50 ± 0.18*	14.34 ± 1.47†	6.05 ± 0.81**†
Caudal end	2.46 ± 0.39	1.21 ± 0.08*	25.20 ± 1.65	16.82 ± 1.87*

rostral end was significantly lower than in the ventral, dorsal and medial regions (Fig. 4), but there was no significant difference in the percentage of FSH-IR cells among different zones of the PD from castrated viscacha. In addition, the percentage of LH-IR cells decreased significantly in the ventral region and at the rostral end and the percentage of FSH-IR cells decreased in the ventral and dorsal regions and at the rostral end of the PD from castrated compared with control viscachas (Figs 3*e–h*, 4).

LH-IR cells and FSH-IR cells expressing nuclear AR were found near blood vessels, in basal positions relative to follicular structures and interspersed between other LH-IR or FSH-IR cells that did not express AR. The percentage of LH-IR/AR-IR and FSH-IR/AR-IR cells did not differ significantly among different zones of the PD from control viscacha. In the castrated group, some LH-IR and FSH-IR cells exhibited hypertrophic-like morphology and negative for AR labelling. The percentage of LH-IR/AR-IR and FSH-IR/AR-IR cells did not differ significantly among different zones of the PD from castrated viscacha. In addition, no significant changes were observed in the percentage of LH-IR/AR-IR cells after castration, whereas a significant decrease in the percentage of FSH-IR/AR-IR cells was observed in all PD zones of castrated compared with control viscachas (Figs 5, 6).

Transmission electron microscopy revealed cells with ultrastructural alterations after castration, mainly differences in the dilatation of endoplasmic reticulum cisternae and scarce secretory granules in the cytoplasm (Fig. 7).

Discussion

Our experimental model, the viscacha (*L. maximus maximus*), exhibits changes in pituitary cell types during the seasonal reproductive cycle in relation to the natural photoperiod. Immunomorphometric studies have revealed that hormone-secreting cells have lower activity during the gonadal regression period (winter, short photoperiod; Filippa *et al.* 2005; Filippa and Mohamed 2006*a*, 2006*b*, 2008, 2010). In addition, the results obtained after melatonin administration correlated with

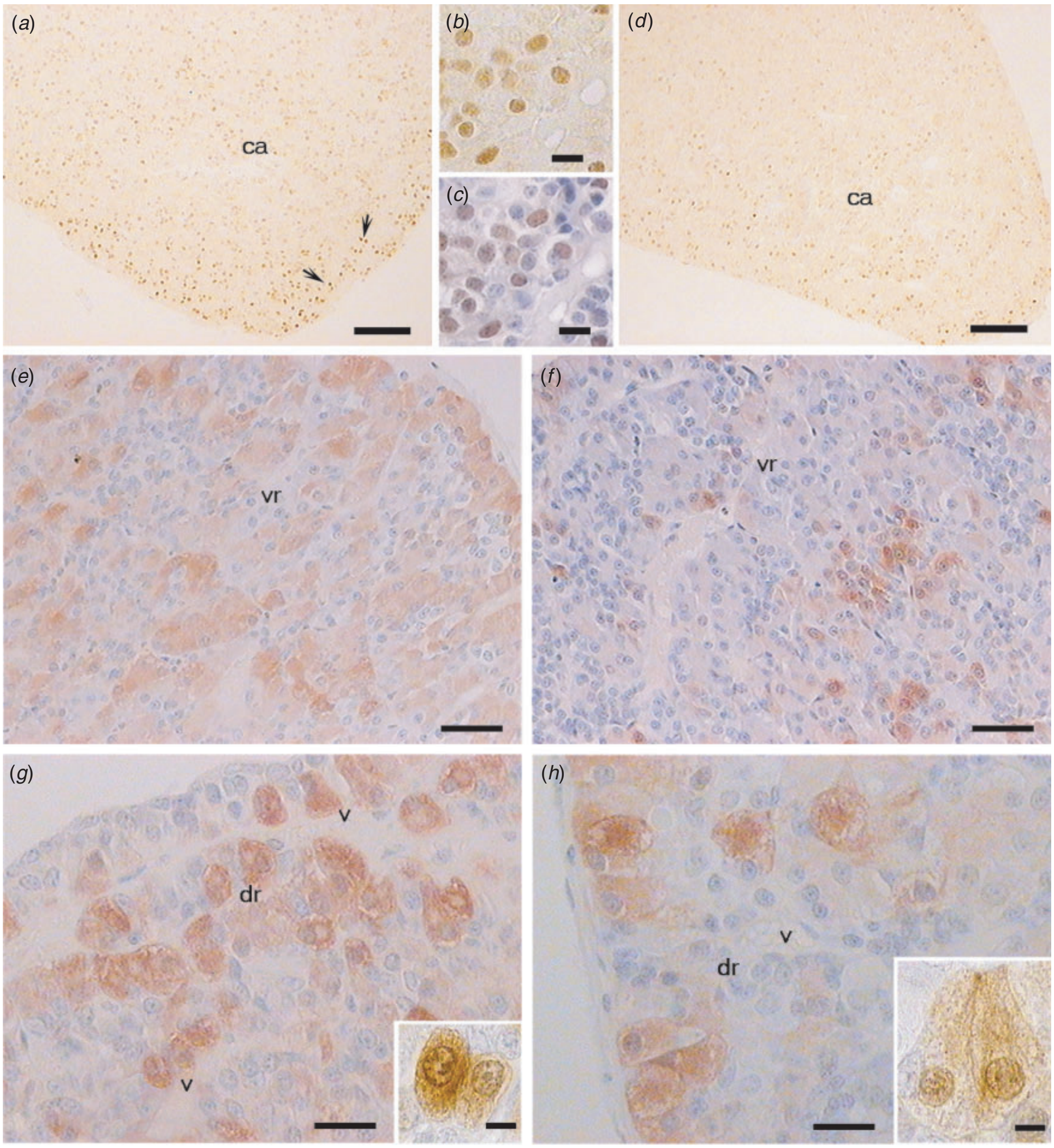

Fig. 3. (*a*) Immunohistochemistry with anti-human (h) androgen receptor (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) serum in the caudal end of pituitaries from control viscachas. Nuclear immunolabelling was copious and intense (arrows) in the control group. Scale bar = 50 μm. (*b*, *c*) Nuclear androgen receptors (ARn) in the pars distalis without (*b*) and with (*c*) haematoxylin staining. Scale bar = 25 μm. (*d*) Androgen receptor immunoreactivity in the caudal end of pituitaries from castrated viscachas. Scale bar = 50 μm. (*e*, *f*) LH-immunoreactive (IR) cells in the ventral region of pituitaries from control (*e*) and castrated (*f*) viscacha. Scale bar = 50 μm. (*g*, *h*) FSH-IR cells in the dorsal region of pituitaries from control (*g*) and castrated (*h*) viscachas. Scale bar = 50 μm. Insets: higher-magnification images of FSH-IR cells from control (*g*) and castrated (*h*) viscachas. Scale bar = 10 μm. v, blood vessels; ca, caudal end; vr, ventral region; dr, dorsal region.

those obtained during the regression period of the annual reproductive cycle, demonstrating the effect of natural photoperiod on pituitary PD cells. During the short photoperiod, when the viscacha are in gonadal regression, high serum levels of melatonin (Fuentes *et al.* 2003) and low serum levels of testosterone serum (Chaves *et al.* 2012) have been reported. Maximum activity of the pituitary–gonadal axis has been observed during the long photoperiod (Muñoz *et al.* 1998; Filippa *et al.* 2005, 2012). The present study into the effects of castration was performed under conditions of a long photoperiod, allowing morphological analysis of the effects of the lack of testicular androgens on pituitary PD cells.

This is the first report on the distribution of AR and their expression in the gonadotrophs; it quantifies changes in

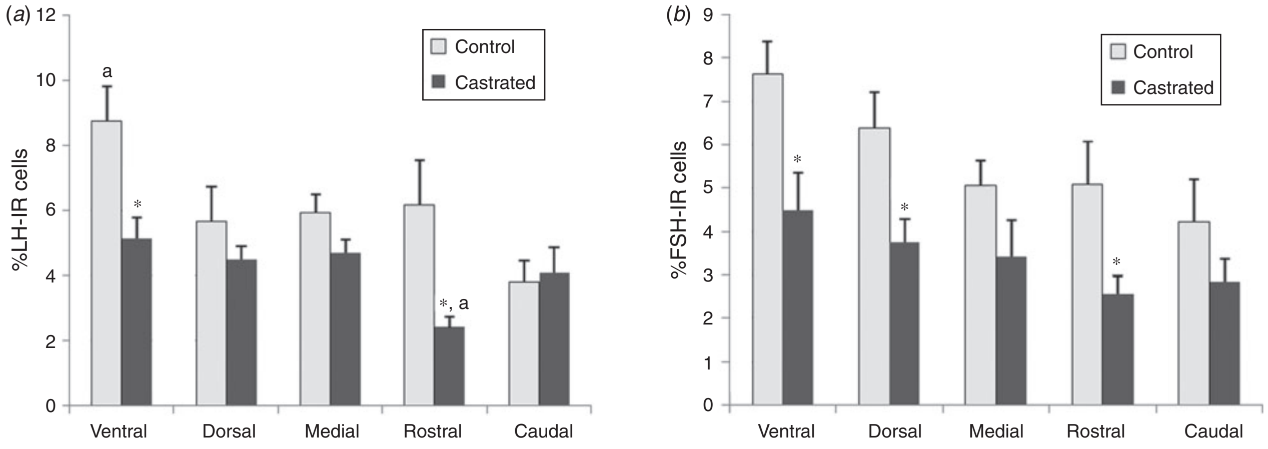

Fig. 4. Percentage of (*a*) LH-immunoreactive (IR) cells and (*b*) FSH-IR cells in each of the different zones of the pituitary pars distalis in control and castrated viscachas. Data are the mean ± s.e.m. *$P < 0.05$ compared with the control group (Student's *t*-test); $^{a}P < 0.05$ for the ventral region vs caudal end in the control group; $^{a}P < 0.05$: rostral end vs ventral, medial and dorsal regions in the castrated group (ANOVA followed by the Tukey–Kramer test).

AR-IR cells and gonadotrophs in the PD of adult male viscacha after castration.

The immunohistochemical results obtained in the present study indicate that the AR are mainly expressed in the nuclei of pituitary PD cells in the viscacha. The ARn-IR and ARc-IR cells decrease significantly after castration in all parenchyma of the PD. Moreover, ARn-IR, LH-IR and FSH-IR cells were regionalised. The percentage of double-immunolabelled cells was similar in the different PD zones of intact animals: the percentage of LH-IR and FSH-IR cells expressing AR was in the range of 45%–66% and 49%–57%, respectively. In castrated animals the percentage of LH-IR cells decreased in the ventral region and rostral end of the PD, whereas the percentage of FSH-IR cells decreased in the ventral and dorsal regions and at the rostral end of the PD. Castration resulted in a significant decrease in the percentage of FSH-IR/AR-IR cells throughout the PD, whereas there was no significant change in the percentage of LH-IR/AR-IR cells in the PD.

Castrated animal models have been used to examine the effects of the withdrawal of circulating androgens on pituitary cells, mainly on the gonadotrophs, due to their involvement in reproductive processes. It has been reported that AR respond to castration in a classic manner (i.e. an increase in ARc and a decrease in ARn) in most species studied (Handa and Resko 1988; Kyprianou and Isaacs 1988; Iqbal *et al.* 1995), but exceptions have also been reported (Choate and Resko 1996). After castration, a decrease in the concentration of pituitary ARn was observed (Pelletier *et al.* 1985; Thieulant and Pelletier 1988), indicating a direct relationship between the immunohistochemical detection of ARn and circulating androgens (Iqbal *et al.* 1995).

The present study describes the localisation of AR in the parenchyma of the PD of viscachas. A significant decrease in ARn and ARc immunolabelling was observed in castrated animals, in accordance with the absence of testicular androgens, is probably that the testicular androgen levels are directly related to the expression of AR in pituitary PD cells.

Some studies have reported morphological, morphometric and ultrastructural variations in pituitary gonadotrophs after castration. In most cases, the gonadotrophs increased during the first months after gonadectomy and decreased with long-term castration (Ibrahim *et al.* 1986; Childs *et al.* 1990). However, it should be noted that the reported variations in LH- and FSH-secreting gonadotrophs are always of different magnitudes, which could be explained by the differential rates of the synthesis or degradation of each hormone; GnRH affects the secretion of FSH and LH differentially (Condon *et al.* 1985). Gonadal peptides, inhibins and FSH-releasing peptides may also contribute to the differential regulation of gonadotropins after castration (Ling *et al.* 1986; Gharib *et al.* 1987). Moreover, it has been reported that small cells may proliferate or be recruited to provide a continued supply of mature gonadotrophs (Childs *et al.* 1990).

In the ventral region and rostral end of the PD of viscacha, LH- and FSH-secreting cells are numerous and a decrease in the percentage of these cells was observed after castration. This demonstrates a reduction of the hormonal deposit, probably due to a greater secretory activity of the cells after castration. The cells containing gonadotropins decreased in the ventral region and rostral end of the PD, whereas FSH-IR cells decreased in the dorsal region, suggesting that LH-IR or FSH-IR cells may be differently regulated by sex steroids depending on the PD zone.

Numerous investigations have attempted to elucidate how androgen regulates gonadotrophs through specific receptors (Spady *et al.* 2004; Scheithauer *et al.* 2008). It has been reported that the pituitary is an androgen target tissue that serves as a potential site of androgen feedback on LH secretion (Schanbacher *et al.* 1987; Handa and Resko 1988). Sharma *et al.* (1990) have demonstrated that androgens maintain the synthesis and molecular composition of pituitary FSH in rats. Attardi *et al.* (1992) have reported that the testis imposes an inhibition on the expression of the genes encoding gonadotropin subunits and that this suppression of some genes varies according to the species studied. In addition, these authors have reported that the pituitary of monkeys may be a source of activin, which

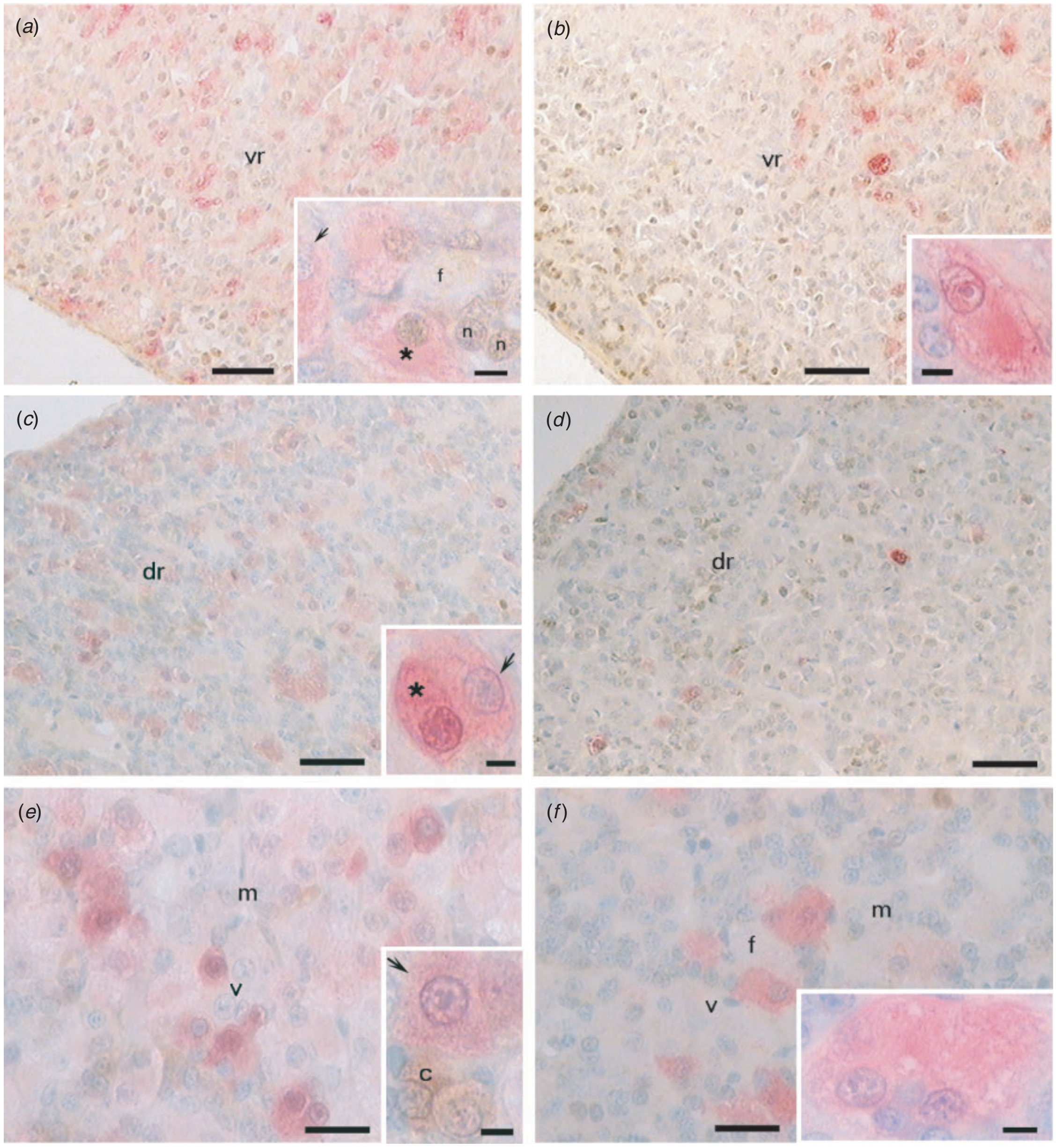

Fig. 5. Double immunolabelling. (*a*) LH-immunoreactive (IR) cells expressing nuclear androgen receptors (AR) in the ventral region (vr) of the pars distalis (PD) from control viscachas. Scale bar = 50 µm. Inset: higher-magnification image of LH-IR/AR-IR cells (*) localised near a follicular structure (f), LH-IR cells that do not express AR (arrow) and nuclear immunolabelling for AR (n). Scale bar = 10 µm. (*b*) LH-IR/AR-IR cells in the vr of the PD from castrated viscachas. Scale bar = 50 µm. Inset: LH-IR cells with hypertrophic-like morphology and negative for AR labelling observed in the PD of castrated animals. Scale bar = 10 µm. (*c*) FSH-IR/AR-IR cells in the dorsal region (dr) of the PD from control animals. Scale bar = 50 µm. Inset: higher-magnification image of FSH-IR cells expressing nuclear AR (*) and FSH-IR cells that do not express AR (arrow). Scale bar = 10 µm. (*d*) FSH-IR/AR-IR cells in the dr of the PD from castrated viscachas. Scale bar = 50 µm. (*e*) FSH-IR/AR-IR cells in the medial region (m) of the PD of the control group. Scale bar = 25 µm. Inset: FSH-IR cells (arrow) and cytoplasmic immunolabelling for AR (c). Scale bar = 10 µm. (*f*) FSH-IR/AR-IR cells in the m of the PD from castrated animals. The number of FSH-IR/AR-IR cells decreased in the dorsal and medial regions in castrated viscachas. Scale bar = 25 µm. Inset: hypertrophic FSH-IR cells negative for AR labelling. Scale bar = 10 µm. v, blood vessels.

may act locally to modulate *FSH gene* expression and secretion. Other researchers have observed that androgens mediated by AR have a negative feedback effect, as evidenced by the inhibition of gonadotropic hormone secretion in the pituitary (Scheithauer *et al.* 2008).

Previous seasonal studies performed in viscacha have demonstrated that reduced activity of LH-secreting cells coincided with lowest values of serum testosterone and LH (Fuentes *et al.* 1991; García-Assef 1996; Filippa *et al.* 2005; Chaves *et al.* 2012). In addition, the maximum levels of serum testosterone

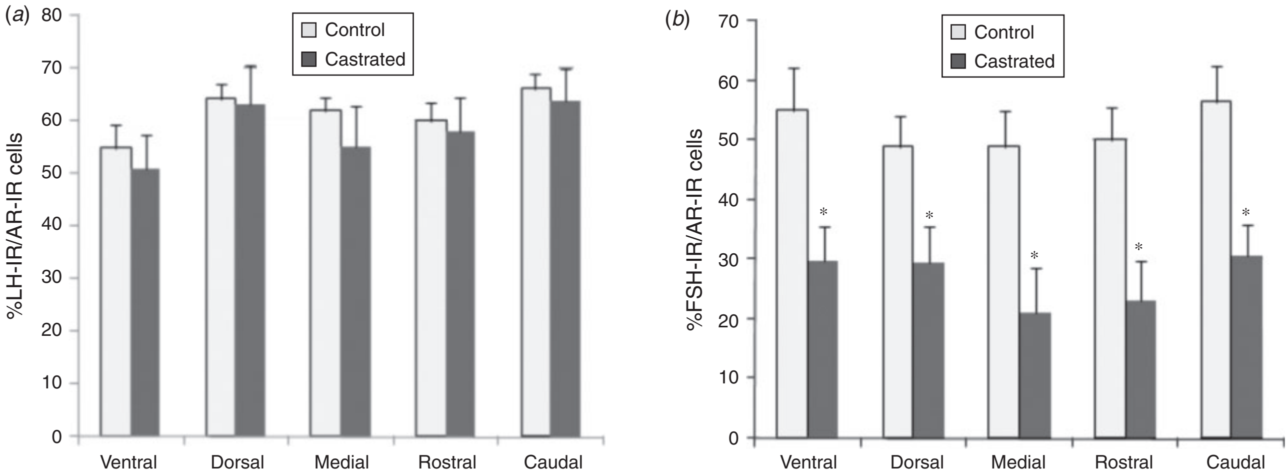

Fig. 6. Percentage of (*a*) LH-immunoreactive (IR)/androgen receptor (AR)-IR cells and (*b*) FSH-IR/AR-IR cells in each of the different zones of the pituitary pars distalis in control and castrated viscachas. Data are the mean ± s.e.m. $*P < 0.05$ compared with the control group (Student's *t*-test).

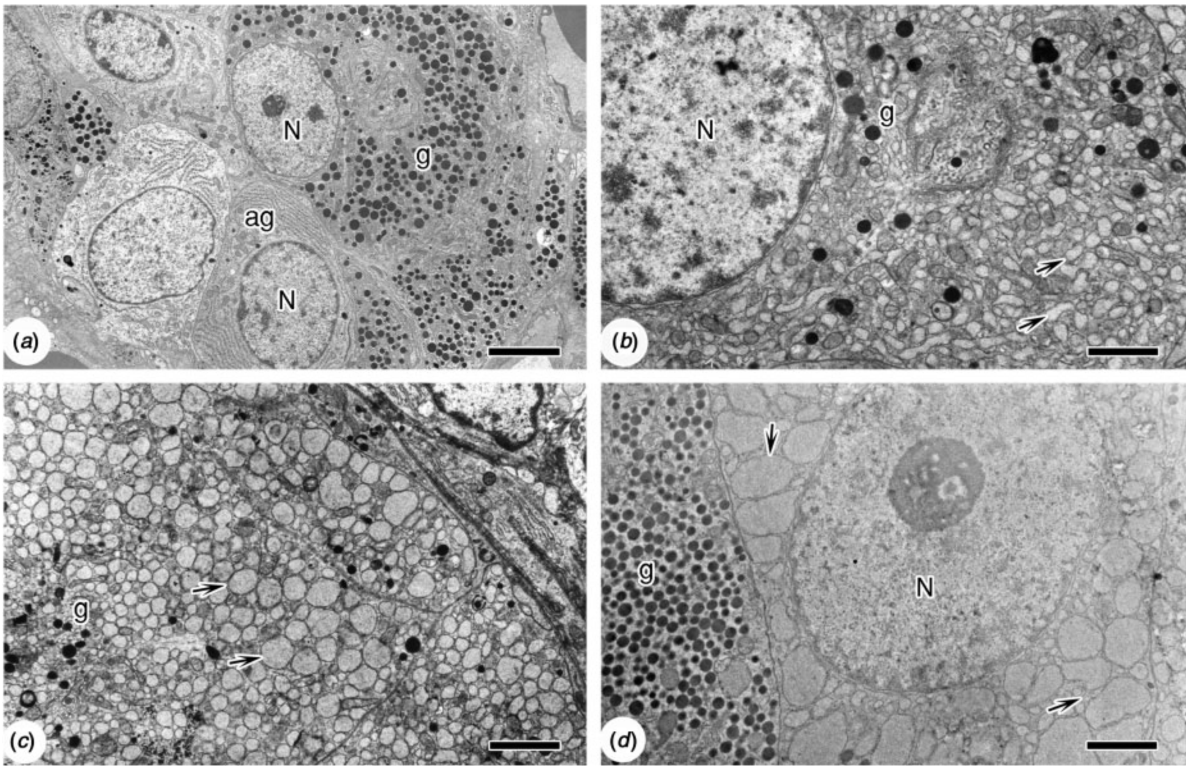

Fig. 7. Conventional transmission electron microscopy. (*a*) Electron micrograph of different cell types in the pituitary pars distalis (PD) of a control viscacha. Granular (g) and agranular (ag) cells of the PD. Scale bar = 5 µm. (*b–d*) Images of PD cells in castrated viscachas showing a progressive dilatation of the endoplasmic reticulum cisternae (arrows) and a decrease in the number of secretory granules (g) in the cytoplasm. Some cells remain granulated (g) without ultrastructural alterations (*d*). Scale bar = 1.75 µm. N, nuclei.

coincided with a decrease in the percentage area occupied by FSH-containing cells in the PD, suggesting that testosterone acts on these cells, directly or indirectly, to regulate their activity (Fuentes *et al.* 1993; Filippa *et al.* 2005). In the present study, we demonstrated a decrease of AR in all PD zones and a reduction in LH-IR cells in certain zones, but there was no decrease in double-labelled LH-IR/AR-IR cells in the PD. Thus, the cells responsible for the decrease in LH immunolabelling after castration are the gonadotrophs that do not express AR, and those cells are localised in the rostral end and ventral region of the PD. In addition, the percentage of FSH-IR cells decreased in three of five PD zones and the percentage of FSH-IR/AR-IR cells decreased significantly throughout the PD. These results suggest that regulation of the activity of FSH-cells is not exclusively related to androgen, and that there are probably other regulatory pathways for these cells in castrated viscachas. In addition, the lack of gonadal androgens mainly affects FSH-containing cells expressing AR. Thus, the results suggest that AR may have different roles in the regulation of gonadotroph activity.

Light and electron microscopy studies of the pituitary of castrated animals have described the castration cells, or signet-ring cells, containing large vacuoles. These cells have a dilated endoplasmic reticulum, resulting in rounded or irregularly shaped vacuoles and dilated cisternae similar to filigree, as well as to the numerical depletion and disappearance of secretory granules, with those remaining localised in the cell periphery (Farquhar and Rinehart 1954; Yoshimura and Harumiya 1965). A notable increase of ultrastructurally altered gonadotrophs was observed 6 months after gonadectomy (Ibrahim *et al.* 1986). Cónsole *et al.* (2001) concluded that a lack of androgen resulted in hypertrophy–hyperplasia of the FSH cells, and hypertrophy of LH-secreting cells, with marked alterations at the ultrastructural level suggestive of a hyperstimulation stage. In the viscacha pituitary, ultrastructural alterations (i.e. dilatation of endoplasmic reticulum cisternae and few secretory granules in the cytoplasm) were observed 6 weeks after castration.

Finally, the present study demonstrated changes in the immunostaining for AR, LH and FSH in castrated adult male viscacha. The colocalisation of AR and FSH is most affected by the lack of gonadal androgens; some gonadotroph subpopulations may exist with different regulatory mechanisms for hormonal synthesis, storage and secretion.

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