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Comparative expression of androgen receptor in the testis and epididymal region of roosters (*Gallus domesticus*) and drakes (*Anas platyrhynchos*)

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Abstract

The androgen receptor (AR) mediates the physiological actions of androgens, which play a crucial role in the maintenance of male reproductive function and fertility. Although the AR distribution pattern is well established in mammalian reproductive organs, information about the AR expression in the testes and epididymal region of birds is still scarce. To better clarify the pattern of AR expression in the avian male tract, we investigated the expression and precise cellular distribution of AR in the testis and epididymal region of roosters and drakes. AR expression was investigated by immunohistochemistry and Western blotting. In the testis, AR was found restricted to the nuclei of Sertoli cells, Leydig cells and some myoid cells in both species. Within the epididymal region, AR was widely expressed in the epithelia of all segments, although with segment specific differences in intensity and cellular distribution. Stronger positivity for AR was found in the principal cells of the epididymal duct, followed by the rete testis epithelium and non-ciliated cells of the distal efferent ductules. Non-ciliated cells of the proximal efferent ductules epithelium showed the lowest immunostaining. Ciliated cells of both segments of the efferent ductules were negative for AR. The connective tissue of roosters presented fewer AR-positive cells when compared with drakes; despite the similar total number of cells in both species. In conclusion, cellular and segment specific differences in AR expression suggest difference in sensitivity to androgens among the ducts composing the epididymal region of roosters and drakes.

Keywords: Androgen receptor; Efferent ductules; Epididymis; Roosters; Drakes

1. Introduction

The androgen receptor (AR) is a member of the nuclear steroid hormone family, which modulates the expression of androgen-dependent target genes (Hiipakka and Liao, 1998). This protein mediates the physiological actions of androgens, i.e., testosterone and dihydrotestosterone, which are essential for maintenance of male reproductive functions and fertility (McLachlan et al., 2002; Dohle et al., 2003). In mammals, androgen is critical for maintenance of the spermatogenesis and regulation of the luminal microenvironment to guarantee the sperm transport, maturation and storage (Vornberger et al., 1994; Robaire and Viger, 1995; Dohle et al., 2003; Hill et al., 2004). In birds, studies concerning the role of androgens in the male reproductive organs are still scarce. It has been shown that in some seasonal breeding avian species there is a positive association between the testosterone plasma levels and testis size (Garamszegi et al., 2005; Denk and Kempenaers, 2006). Nevertheless, direct or indirect inhibitory effect of testosterone to the growth of testicular components has also been described (Purcell and Wilson, 1975).

Comparing with the testis, lesser is known about the androgen response in other segments of the avian male genital tract. Actually, it was not until 2006 that the full-length androgen receptor cDNA of chicken, one of the avian species most extensively used for commercial and experimental

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purposes, was cloned (Katoh et al., 2006). Similarly, it was just recently that the pattern of testosterone secretion was determined in this species (Vizcarra et al., 2004). Therefore, it is not surprising that the distribution, function and mechanism of regulation of the androgen responsive system in male reproductive organs of chickens as well as of other avian species remain poorly understood. To address these issues, it becomes important first to clearly determine the androgen-target cells within the testis and genital tract. In this regard, the cellular expression of AR in the avian testis is still a matter of debate (Nastiuk and Clayton, 1994; Shanbhag and Sharp, 1996). In chicken, AR protein has been found in Leydig cells (Shanbhag and Sharp, 1996), whereas in the seasonal breeding canary the AR transcript has been described in Sertoli cells but not in Leydig cells (Nastiuk and Clayton, 1994). These results point out for possible differences in the pattern of AR cell distribution among species and/or breeding pattern.

Differing from mammals, the male excurrent ducts of avian species are characterized by the occurrence of prominent efferent ductules, which composes approximately 50% of the epididymal region, contrasting with a short and non-differentiated epididymal duct (Lake, 1957; Aire, 1979; Oliveira et al., 2007). The epididymal region also includes the extratesticular rete testis and a short connecting duct, which connect the distal efferent ductules with the epididymal duct. Concerning the AR expression in the rooster epididymal region, one report has been published; however, the authors have not detailed the cellular and regional expression of this receptor (Shanbhag and Sharp, 1996). Furthermore, to the best of our knowledge, complete information about the AR distribution in the epididymal region of drakes is not available. Therefore, owing to better clarify the pattern of AR expression in the avian male tract, in the present study we aimed to investigate the expression and precise cellular distribution of AR in the testis and each segment composing the epididymal region of two species, roosters and drakes.

2. Materials and methods

2.1. Animals

The investigation was performed on the epididymal region of adult crossbreed roosters (*Gallus domesticus*) and mallard drakes (*Anas platyrhynchos*), during breeding phase. The animals were obtained from domestic and commercial sources and housed at the facilities of the Federal University of Minas Gerais, MG, Brazil. The animals were maintained in natural conditions of light, humidity and temperature, and were allowed free access to water and commercial food (Socil-III/Guyomarc'H; Belo Horizonte, MG, Brazil). The principles of research involving animals followed those expressed in the 'Princípios éticos para o uso de animais em experimentação', advocated by the local ethical committee (Comitê de ética em pesquisa), published by the Federal University of Minas Gerais—UFMG (http://www.ufmg.br/bioetica/cetea/).

2.2. Tissue preparation

The roosters and drakes were weighted, anesthetized (i.p. sodium pentobarbital 50 mg/kg body weight), and perfused intracardially with 10% neutral buffer formalin. After fixation, the epididymal regions were isolated from the testis and fragments of tissue were embedded in paraffin, sectioned at 5.0 μm and used for immunohistochemistry.

2.3. Immunohistochemistry

Androgen receptor (AR) expression was localized in the epididymal region by using immunohistochemistry, following previous protocol (Oliveira et al., 2007). Staining was performed in two different sets to confirm the results. Sections were dewaxed in xylene, rehydrated through a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS) and then blocked for endogenous peroxidase by incubation with 0.6% H_2O_2 in methanol for 30 min. The sections were subjected to antigen retrieval procedure by microwaving in 0.01 M sodium citrate buffer pH 6.0. After washing in PBS, the avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA, USA). Additional washing in PBS was performed before the next 1 h incubation in 10% normal goat serum. The sections were incubated overnight at 4 °C with the diluted (1:500) primary rabbit anti-rat polyclonal AR antibody (AR Ab-2—Labvision Co., Fremont, CA, USA) raised against a synthetic peptide derived from the N-terminus of rat androgen receptor. For negative control, the sections received PBS in place of the primary antibody. After washing in PBS, the sections were exposed to 10% normal goat serum for 1 h before incubation with a goat anti-rabbit biotinylated secondary antibody (Dako, Carpinteria, CA, USA), used at 1:50 dilution. The sections were then incubated with avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA, USA) for 30 min. To visualize the immunoreaction, sections were immersed in 0.05% 3,3′-diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. The reaction was monitored microscopically and stopped by immersion in distilled water, as soon as a brown color staining was visualized. Sections were lightly counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

2.4. Scoring of immunostaining intensity

AR immunostaining intensity was quantified by computer-assisted image analysis, based on previously reported protocol (Dornas et al., 2007). Images from five different areas of the rete testis, proximal and distal efferent ductules as well as connecting/epididymal ducts of each animal were taken by using a Nikon Eclipse E600 microscope (Nikon Co. Melville, NY, USA). Due to difficulties to distinguish the connecting ducts from the epididymal duct, these segments were considered together. Digital images were processed by using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). After conversion to the grayscale mode and inversion, the images were exported to Image-Tool software (University of Texas Health Sciences Center, San Antonio, TX, USA), for quantitative analysis. For this purpose, 25 nuclei of epithelial non-ciliated cells of the proximal and distal efferent ductules as well as principal cells of the epididymal duct were measured. The nuclei were traced and the pixel intensity was determined for the traced areas. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells. Final pixel intensity was calculated by subtracting the values detected in labeled nuclei from the background.

Considering that the staining intensity was not measured in all cell types composing the duct of the epididymal region, a qualitative scoring of immunostaining was also performed on the basis of nuclear staining, which was classified as negative (no staining), weak (+), moderate (++) or strong (+++). Nuclei were reported as negative when the staining did not differ from the negative control sections. Staining for AR in epididymal duct was defined as a baseline strong staining.

2.5. Western blotting

The antibody used in this study has already been shown to be specific to drake androgen receptor (Oliveira et al., 2007), but its specificity against rooster androgen receptor has not been reported. Therefore, Western blotting analyzes were performed based on previously described protocol

(Oliveira et al., 2007), to confirm the specificity of the antibody used. In summary, epididymal regions from rooster and drakes perfused with a 0.75% saline solution were dissected out, rinsed vigorously in PBS and frozen in liquid nitrogen. Frozen fragments (100 mg) were macerated in dry ice and resuspended in 750 μl of sample buffer under reducing conditions. After boiling for 5 min, the samples were subjected to continuous electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). The separated proteins were transferred to nitrocellulose membrane and blocked with 10% normal goat serum for 1 h at room temperature. The membrane was incubated with rabbit anti-rat polyclonal antibody against AR (AR Ab-2—Labvision Co., Fremont, CA, USA) diluted 1:500 for 1 h. After washing with PBS–Tween 0.05%, the blot was incubated in a biotinylated secondary antibody goat anti-rabbit (Dako, Carpinteria, CA, USA), used at 1:2000 dilution. The membrane was then incubated with the avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA, USA) for 30 min. After several washes, the reaction was developed by the addition of 0.1% 3,3′-diaminobenzidine in PBS containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H_2O_2. The reaction was stopped with deionized water.

2.6. Morphometry

Quantitative studies of the total cells and those immunolabeled to AR in the connective tissue of the epididymal region of roosters and drakes were performed by using classic stereological methodology (Weibel et al., 1969). For each case, the number of cells was obtained by counting all the stromal cells as well as the cells positive to AR, in 15 randomly selected sections of constant areas (μm^2), using a grid of 100 intersections. Then, the proportion of cells/mm^2 was calculated.

2.7. Statistical analysis

Differences in AR expression among segments of the epididymal region of roosters and drakes were analyzed by Kruskal–Wallis one-way analysis of variance. The post-hoc Tukey test was used for multiple comparisons between segments. In the case of the morphometry of the connective tissue, differences in the number of AR-positive cells as well as the total cells in the stromal tissue were compared using the Student's *t*-test. Differences were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Western blotting

The specificity of the androgen receptor antibody for use in avian tissue was confirmed by Western blotting assay. The AR-antibody recognized a single band of about 100 kDa in the epididymal region of both species studied (Fig. 1), which is in agreement with the AR molecular weight previously described for chickens and drakes (Yoshimura et al., 1993; Oliveira et al., 2007).

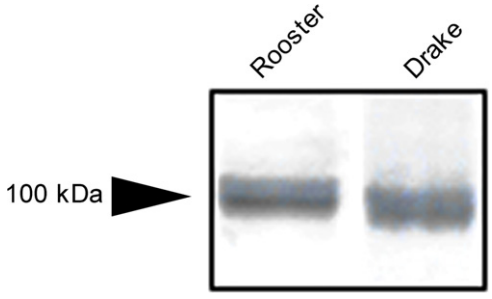

Fig. 1. Western blotting analysis of androgen receptor (AR) expression in the epididymal region of roosters and drakes. The antibody to AR detected a single protein band of about 100 kDa in the epididymal region tissue of both species.

3.2. Regional distribution of AR protein

The testis and the epididymal region of both roosters and drakes showed positivity to androgen receptor protein, which was cell and segment specifics. The immunoreactivity to AR was noted as a nuclear staining seen in somatic cells of the testis (Fig. 2), as well as epithelial cells and some connective tissue cells surrounding the ducts composing the epididymal region (Figs. 3–5). The intensity of the epithelial staining in the epididymal region varied depending on the segment considered: stronger positivity was found in the epididymal duct, followed by the rete testis and distal efferent ductules (Table 1, Figs. 3 and 4). Proximal efferent ductules showed the lowest immunoreactivity among the excurrent ducts. No immunolabeling was observed in negative control sections (Figs. 2 and 3 inserts).

3.2.1. Testis

The testes of roosters and drakes were formed by numerous convoluted seminiferous tubules, which contained the spermatogenic and Sertoli cells, surrounded by a thin tunica propria. The interstitial tissue was scarce and contained the Leydig cells and blood vessels. AR was expressed in the Sertoli cells, Leydig cells and some myoid cells (Fig. 2), as well as in the vascular endothelial cells of both species analyzed. Immunostaining was not detected in spermatogenic cells.

3.2.2. Epididymal region

The avian epididymal region consisted of the extratesticular rete testis, proximal and distal efferent ductules, followed by the connecting and epididymal ducts, all involved by abundant connective tissue. All these segments showed positivity for AR in both rooster and drake (Table 1, Figs. 3 and 4), as following:

Rete testis. The cuboidal epithelial cells lining the rete testis of both rooster and drake were moderately positive for AR (Fig. 3a and b). Macrophages found at the lumen of this segment were weakly immunoreactive (inserts Fig. 3a and b).

Proximal efferent ductules. Non-ciliated cells of the rooster efferent ductules showed intermittent positivity for AR, being negative in some cells (Fig. 3c). In drakes, the epithelial non-ciliated cells of the efferent ductules were weakly positive for AR (Fig. 3d). Ciliated cells were negative for AR in both species.

Distal efferent ductules. Similar to proximal efferent ductules, ciliated cells of the distal ductules were not stained for AR. In both species, the non-ciliated cells were moderately positive for this receptor (Fig. 3e and f).

Connecting and epididymal duct. In roosters and drakes, the principal cells were strongly positive for AR. Conversely, the basal cells were weakly and intermittently

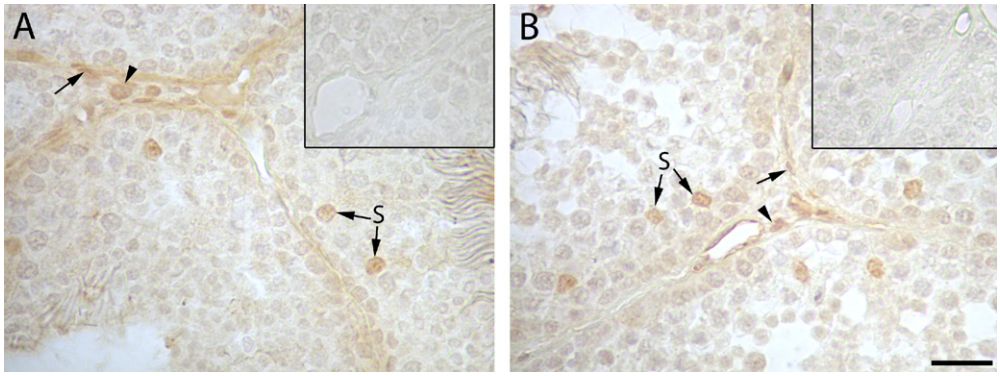

Fig. 2. Androgen receptor (AR) expression in the testis of roosters (A) and drakes (B). AR staining was found in the Sertoli cells (S), Leydig cells (arrowheads) and myoid cells (arrows) in both species. The inserts in (A) and (B) show the negative controls. Bar = 20 μm.

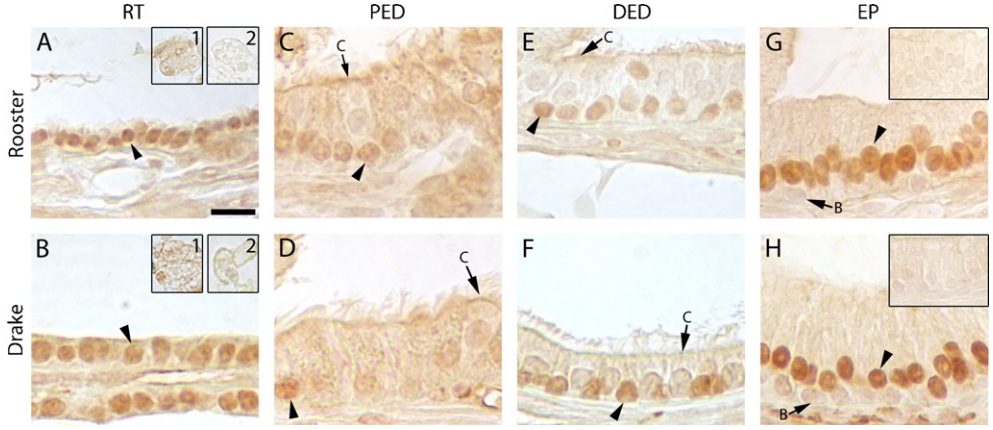

Fig. 3. Androgen receptor (AR) expression in the epididymal region of roosters (A, C, E and G) and drakes (B, D, F and H). (A and B) Extratesticular rete testis (RT) presented AR-positive epithelial cells (arrowheads). Inserts 1 show macrophages positive to AR, whereas inserts 2 show the negative controls. (C–D) Proximal efferent ductule (PED) showed positivity for AR in the non-ciliated cells (arrowheads) which contrasted with negative ciliated cells (C). (E–F) Distal efferent ductule (DED) with moderately immunostained non-ciliated cells (arrowheads). Ciliated cells were negative (C). (G–H) Epididymal duct (EP) showed strong positivity to AR in the principal cells (arrowheads) whereas basal cells (B) were weakly positive or negative. Inserts show the negative controls. Bar in $A = 20$ μm.

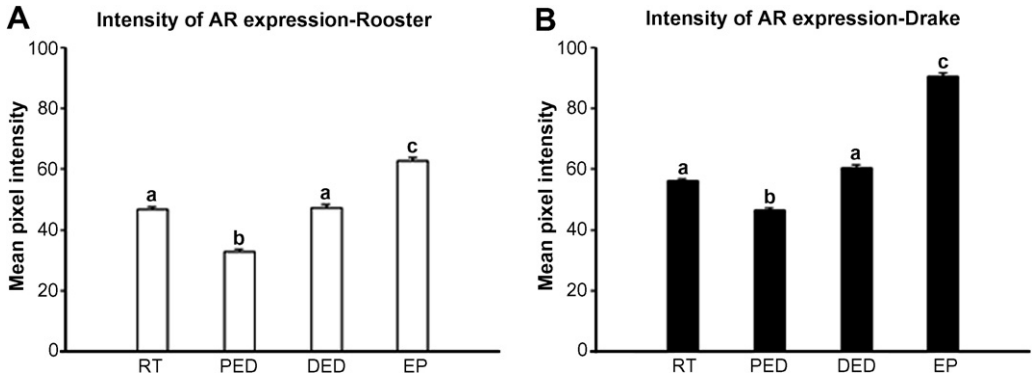

Fig. 4. Quantification of immunohistochemistry for androgen receptor in the epithelium of extratesticular ducts of roosters (A) and drakes (B). a, b, and c indicate differences statistically significant ($P \leqslant 0.05$) among the segments analyzed. Values are represented as means ± SEM; $n = 04$. RT, rete testis; PED, proximal efferent ductules; DED, distal efferent ductules; EP, epididymal duct.

immunostained in drakes, and negative at all in rooster connecting and epididymal ducts (Fig. 3g and h).

Connective tissue. The connective tissue cells were just weakly stained throughout the excurrent ducts of roosters, whereas they were moderately positive for AR in drakes. Compared to drakes, fewer cells of the connective tissue were positive for AR in the epididymal region of roosters. As shown by the morphometrical study, the number of

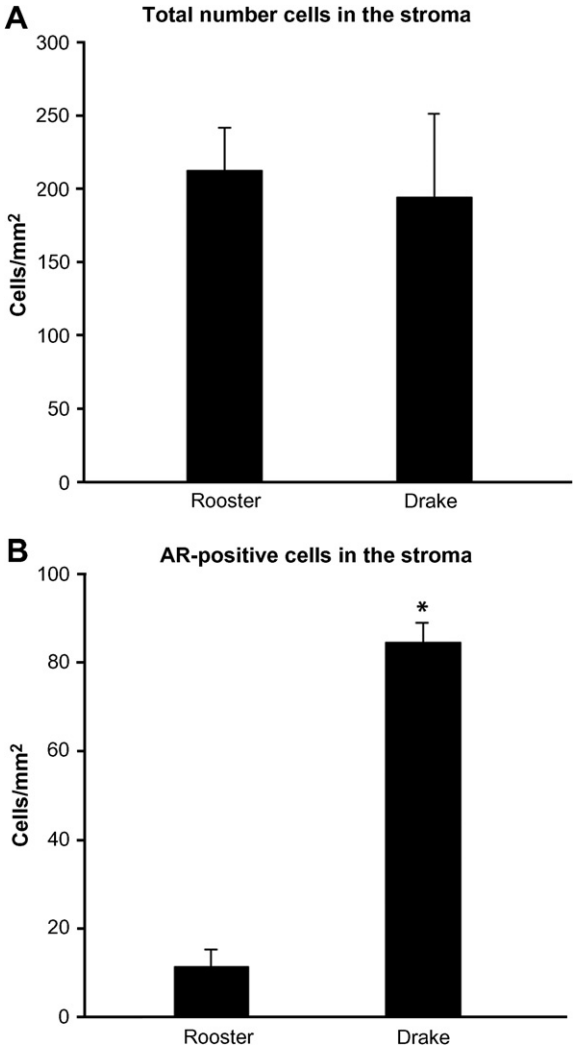

Fig. 5. Quantification of total cells (A) and AR-positive cells (B) in the connective tissue of the epididymal region of roosters and drakes. Values represent mean ± SEM; $^{*}P \leqslant 0.05$. $n = 04$.

Table 1
Scoring of the immunostaining intensity for androgen receptor (AR) in the ducts composing the epididymal region of roosters and drakes

	AR	
	Rooster	Drake
Rete testis		
Epithelial cells	++	++
Macrophage-like cells	+	+
Proximal efferent ductules		
Epithelial non-ciliated cells	+/−	+
Epithelial ciliated cells	−	−
Distal efferent ductules		
Epithelial non-ciliated cells	++	++
Epithelial ciliated cells	−	−
Connecting and epididymal ducts		
Epithelial principal cells	+++	+++
Epithelial basal cells	−	+/−
Connective tissue	+/−	++

Score were as follows: −, negative; +/−, intermittent staining; +, weak staining; ++, moderate staining; +++, strong staining.

cells positive to AR was about 8-fold greater in drakes in comparison to roosters, despite both animals presented similar number of total cells in the stroma (Fig. 5). Vascular endothelium showed positivity for AR in both species.

4. Discussion

In this study we compared the immunolocalization of androgen receptor protein in the testis and epididymal region of roosters and drakes. AR was shown to be restricted to the somatic cells in the testis but widely distributed in the segments composing the epididymal region, although with specific regional pattern of expression along the ducts. Great differences related to the amount of AR-positive cells in the connective tissue of the epididymal region were found between both species analyzed.

Testicular distribution of AR was found in the somatic cells, namely Sertoli cells, Leydig cells and myoid cells, in both roosters and drakes. Previous study in chicken has described AR protein just in Leydig cells (Shanbhag and Sharp, 1996). Conversely, in the canary, the AR transcript has been described in Sertoli cells but not in Leydig cells (Nastiuk and Clayton, 1994). Unlike these previous findings, our result shows a wider distribution of AR in the testis of both species investigated, which is in complete agreement with those found for mammals (Ruizeveld de Winter et al., 1991; Suárez-Quian et al., 1999; Zhu et al., 2000; Zhou et al., 2002). Little is known about spermatogenesis in birds, especially in regard of hormonal regulation of this process. In terms of androgen responsivity, our findings suggest that the regulation of spermatogenesis in roosters and drakes may share similarity with mammals as this hormone appear to influence testicular somatic cells, rather than acting directly on the germ cells (Johnston et al., 2001; Tan et al., 2005).

The occurrence of AR in the efferent ductules and epididymal ducts of roosters and drakes is in agreement with previous findings for avian species (Shanbhag and Sharp, 1996; Nishizawa et al., 2002; Yoshimura and Kawai, 2002), as well as mammals (Roselli et al., 1991; Goyal et al., 1997; Zhu et al., 2000). However, the present result adds to past studies in birds by showing that the rete testis is positive for AR and may also be a target for androgens. Moreover, our data shows for the first time that there is a clear difference in expression of AR along the ducts, suggesting that the proximal efferent ductules may be the least sensitive, whereas the epididymal duct is potentially the most sensitive to androgen among the segments of the epididymal region in both species analyzed. Thus, specific regional functions, as protein expression, fluid reabsorption (Bahr et al., 2006), as well as sperm concentration and maturation (Esponda and Bedford, 1985; Zaniboni et al., 2004) in the avian epididymal region may be differentially regulated by androgens.

The difference in AR expression found between the proximal and distal segments of the efferent ductules favors previous data showing that, despite the common name, these avian male tract segments arise from different embry-

onic origins (Budras and Sauer, 1975; Budras and Meier, 1981) and presents remarkable morphological and functional differences (Aire, 1980, 2000; Holsberger et al., 2002; Aire et al., 2004; Clulow and Jones, 2004). Based on the weak to negative expression of AR in the proximal efferent ductules, it is possible to speculate that instead of androgen this segment may be regulated by other factors. On this sense, there are convincing data showing that in mammal species the efferent ductule is a segment of the male tract more sensitive to estrogen than androgen (Hess et al., 1997; Oliveira et al., 2002; Hess, 2003). Therefore, estrogens would be one reasonable candidate to regulate the avian proximal efferent ductules as well. In line with this interpretation, the proximal efferent ductules of roosters exhibited strongest positivity for P450 aromatase, the enzyme responsible for conversing testosterone to estradiol (Kwon et al., 1995). Similarly, this segment showed high expression of estrogen receptor ERα (Kwon et al., 1997). It is also noteworthy that the proximal efferent ductules were more sensitive to exposure to Roundup, an herbicide with activity of aromatase inhibition (Richard et al., 2005; Oliveira et al., 2007). Further investigation addressing regional differences in the distribution of estrogen receptors, especially ERβ, in the avian epididymal region would be helpful to substantiate the assumption that estrogen may be the major regulator of the proximal efferent ductules.

Principal cells of the epididymal duct were shown to have the greatest expression of AR, compared to the other segments of the epididymal region, both in roosters and drakes. This result is in agreement with previous findings for the counterpart mammalian epididymis (Roselli et al., 1991; Goyal et al., 1997; Zhu et al., 2000; Yamashita, 2004), indicating that this male tract segment is a major androgen target across species. There was difference among positivity of epididymal basal cells to AR when roosters and drakes were compared. In drakes, the epididymal basal cells were weakly immunostained for AR, which is in agreement with the pattern found for mammals (Goyal et al., 1997; Zhu et al., 2000). The epididymal basal cells of roosters appeared predominantly negative for AR. Previous studies in avian epididymal ducts have not addressed differences in the AR expression between principal and basal cells (Shanbhag and Sharp, 1996; Nishizawa et al., 2002; Yoshimura and Kawai, 2002). However, favoring our results, a weaker and sometimes intermittent staining for AR has been found in basal cells of the epididymis of several mammal species (Zhu et al., 2000; Parlevliet et al., 2006; Pearl et al., 2006).

The connective tissue of the epididymal region of drakes and roosters showed an unexpected and significant difference in number of cells positive for AR. We do not have a clear explanation for this fact. However, one possibility is that the difference may be related to divergences in the pattern of breeding cycle, as drakes have marked breeding periods along the year (Donham, 1979; Haase, 1983), whereas domestic fowl presents a less conspicuous annual breeding cycle (Lofts and Murton, 1968; Sexton, 1983). Testosterone is a potent physiological factor controlling seasonal plasticity in avian tissue (Tramontin et al., 2003; Garamszegi et al., 2005). Therefore, it is possible that the great number of positive AR cells found in the stroma of the epididymal region of the drakes may be mediating and guaranteeing the main functions of the components of this region during breeding phase.

In conclusion, besides the testis, AR was found widely expressed along the segments of the epididymal region of roosters and drakes; however, with cell and segment specific differences in intensity of receptor expression, suggesting different sensitivity to androgens.

Acknowledgments

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