

# Roosters affected by epididymal lithiasis present local alteration in vitamin D3, testosterone and estradiol levels as well as estrogen receptor 2 ( $\beta$ ) expression

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## Abstract

Epididymal lithiasis is a reproductive dysfunction of roosters that is associated with loss of fertility and is characterized by the formation of calcium stones in the lumen of the efferent ductules of the epididymal region. The efferent ductules of birds are responsible for the reabsorption of the fluid coming from the testis as well as luminal calcium. It has been hypothesized that the epididymal stone formation may be related to the impairment of local fluid or calcium homeostasis, which depends on hormones such as estradiol ( $E_2$ ). Therefore, this study aimed to investigate possible alterations in the expression of ER $\alpha$  (ESR1) and ER $\beta$  (ESR2) in the epididymal region of roosters affected by epididymal lithiasis. The study was performed by immunohistochemistry and western blotting assays. In addition, the concentrations of  $E_2$ , vitamin D3, and testosterone, which are also key hormones in maintenance of calcium homeostasis, were determined in the plasma and epididymal region, by ELISA. It was observed that ESR2 expression is increased in all segments of the epididymal region of affected roosters, whereas ESR1 levels are not altered. Moreover, the hormone concentration profiles were changed, as in the epididymal region of roosters with lithiasis the  $E_2$  levels were increased and vitamin D3 as well as testosterone concentrations were significantly decreased. These results suggest that a hormonal imbalance may be involved with the origin and progression of the epididymal lithiasis, possibly by affecting the local fluid or calcium homeostasis.

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## Introduction

Epididymal lithiasis is a reproductive dysfunction characterized by the formation of luminal stones rich in calcium in the rooster's epididymal region (Janssen *et al.* 2000, Mahecha *et al.* 2002, Oliveira & Oliveira 2011). Animals affected by this anomaly present severe testicular and epididymal alterations. Testicular damage includes dilation of seminiferous tubules, sloughing of the seminiferous epithelium, and increase in Leydig cell frequency in the interstitial tissue (Janssen *et al.* 2000, Mahecha *et al.* 2002, Oliveira *et al.* 2008). The testicular alterations result in reduced fertility in affected animals (Janssen *et al.* 2000). Interestingly, the reduction in fertility was observed after natural and even artificial insemination with equivalent numbers of sperm obtained from the semen of affected animals (Janssen *et al.* 2000). This result suggested that adverse effects of epididymal lithiasis on fertility could be attributed to alterations in the production of sperm at the testicular level, but also possibly as a consequence of impaired

maturation as they traverse the epididymal region. In birds, the epididymal region is composed of the rete testis, proximal and distal efferent ductules and epididymal duct (Aire 1979a, Aire & Soley 2000, Oliveira *et al.* 2007), which are also affected by the presence of the stones (Janssen *et al.* 2000, Mahecha *et al.* 2002, Oliveira *et al.* 2008).

The efferent ductules are the most affected segment of the rooster's genital tract in animals with epididymal lithiasis (Janssen *et al.* 2000, Mahecha *et al.* 2002). Besides the fact that the formation of the calcium stones is restricted to the efferent ductules, epithelial injury as well as the occurrence of frequent mononuclear cell infiltrates adjacent to affected ductules are common features in affected animals (Janssen *et al.* 2000, Mahecha *et al.* 2002, Boltz *et al.* 2004, 2006, Jackson *et al.* 2006, Oliveira *et al.* 2008). In birds, the efferent ductules compose up to 60% of the epididymal region (Aire 1979b, Oliveira *et al.* 2007). It has been shown that these ductules are responsible for testicular fluid and calcium reabsorption, an essential function involved in

sperm concentration and maturation (Clulow & Jones 1988, 2004).

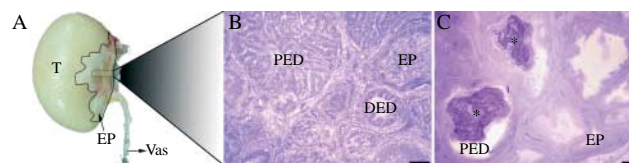
It is well known that the fluid reabsorptive function of the mammalian efferent ductules are under the influence of estrogen and its receptors ESR1 and ESR2, that are highly expressed within the epithelial cells of these ductules (Goyal *et al.* 1997, Hess *et al.* 1997, Nielsen *et al.* 2001, Zhou *et al.* 2001, Nie *et al.* 2002, Oliveira *et al.* 2002, 2005, Picciarelli-Lima *et al.* 2006, Joseph *et al.* 2011). In addition to the regulation of fluid transport, estrogens also participate in the maintenance of calcium homeostasis in several tissues, including in birds, by regulating the expression and activity of proteins involved in the calcium homeostasis (Hoenderop *et al.* 2005, de Matos 2008). In roosters, the relevance of estrogen action in the epididymal region has not been determined. Previous studies have shown that P450 aromatase, the enzyme responsible for estrogen production (Kwon *et al.* 1995), as well as estrogen receptor (ER), are present in the rooster epididymal region (Kwon *et al.* 1997). However, a differential expression of ESR1 and ESR2 has recently been described for this species (Oliveira *et al.* 2011). It was shown that ESR2 is widely expressed within the epididymal region, whereas ESR1 was mostly found in the distal efferent ductules, suggesting that, as in mammals, these ductules may be important targets for estrogen action in roosters (Oliveira *et al.* 2011).

It has been hypothesized that the epididymal stone formation may be related to impairment of fluid or calcium transepithelial transport in the efferent ductules (Oliveira *et al.* 2008, Oliveira & Oliveira 2011). Therefore, based on the dual role of estrogens and their receptors in the transepithelial transport of fluid and calcium, this study aimed to investigate the expression of ESR1 and ESR2 in the epididymal region of roosters affected by epididymal lithiasis as well as the concentrations of estrogens in the plasma and epididymal region. In addition, we also addressed the levels of vitamin D3 and testosterone, considering that previous studies have shown alterations in both vitamin D3 receptor (VDR) and androgen receptor in the epididymal region of roosters affected by lithiasis (Oliveira *et al.* 2008).

## Results

### Epididymal region

The epididymal region of roosters lies closely attached to the testis and is formed by the extratesticular rete testis, proximal, and distal efferent ductules as well as connecting and epididymal ducts (Fig. 1). In males affected by the epididymal lithiasis, stones were visible macro- and microscopically within the lumen of efferent ductules, especially in the proximal efferent ductules that also presented loss of epithelial folding (Fig. 1).



**Figure 1** The epididymal region of roosters. (A) Macroscopical view of the testis and epididymal region (highlighted area). (B) The epididymal region of non-affected animals showing proximal efferent ductules with highly folded epithelium (PED), distal efferent ductules (DED), and epididymal duct (EP). (C) Epididymal region of roosters affected by epididymal lithiasis showing luminal stones (\*) and loss of epithelial folding in proximal efferent ductules (PED). Epididymal duct (EP) shows no evident alterations. Bar in B and C = 100 µm. T, testis; EP, epididymal region; Vas, deferent duct.

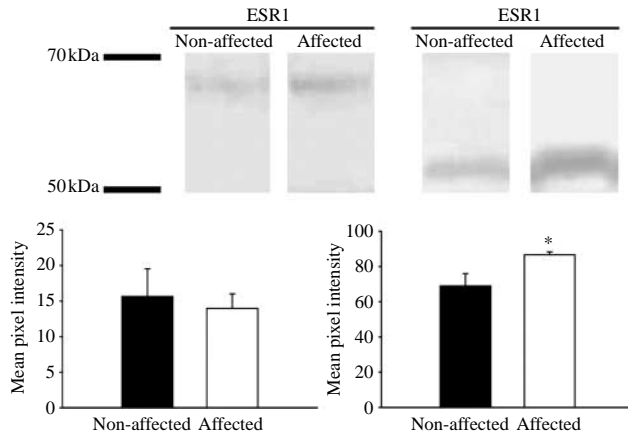
The efferent ductules compose the majority of the volume (23%) of the ducts in the epididymal region of the roosters, followed by the rete testis (16%) and connecting ducts/epididymal duct (10%). A great proportion of the region is formed by connective tissue (51%). No alterations in the proportion of the epididymal region components were found between animals non-affected and affected males.

### ESR1 and ESR2 detection

Western blotting assays for ESR1 and ESR2 detected specific positive protein bands of 67 and 54 kDa respectively (Fig. 2). These results are in agreement with previous published data for these receptors in birds (Gonzalez-Moran *et al.* 2008, Oliveira *et al.* 2011). A significant increase (26%) in ESR2 expression was observed in the epididymal region of roosters affected by epididymal lithiasis compared with non-affected animals (Fig. 2B). On the other hand, ESR1 expression was not statistically significant between the groups (Fig. 2A).

The highest ESR1 labeling was found in the nuclei of non-ciliated cells of the distal efferent ductules, followed by principal and basal cells in the epididymal duct (Fig. 3A–C). On the other hand, the non-ciliated cells of the proximal efferent ductules were intermittently positive for ESR1. Cells of the connective tissue and ciliated cells of the efferent ductules were not immunolabeled for this protein. Compared with non-affected animals, roosters affected by epididymal lithiasis did not show evident alterations in ESR1 (Fig. 3D–F).

ESR2 was widely expressed in the epididymal region of roosters, as all epithelial cell nuclei as well as connective tissue cells were positive for this protein (Fig. 4A–C). In the efferent ductules, ESR2 immunoreaction was observed in both non-ciliated and ciliated cells lining the epithelium, as well as in principal and basal cells of the epididymal duct. There were no significant differences in ESR2 immunostaining between the segments of the epididymal region in non-affected animals. Compared with non-affected roosters, animals affected by lithiasis presented increased ESR2 labeling in



**Figure 2** Western blotting analysis of ESR1 and ESR2 in the epididymal region of roosters affected and non-affected by epididymal lithiasis (A). Graphical representation of the image analysis of the ESR1 (B) and ESR2 (C) western blots.  $n=5$ ;  $*P\leq 0.05$ .

the epithelium of all segments analyzed. On the other hand, the immunoreaction for this receptor in the connective tissue cells was similar in both groups (Fig. 4D–F). In the proximal efferent ductules, ESR2 levels were higher in the non-ciliated cells (38%) and in ciliated cells (44%); whereas in the distal efferent ductules the increase in staining was in the magnitude of 44 and 45% respectively (Fig. 4G and H). In addition, principal cells lining the epididymal duct showed an increase of 41% in the immunostaining for this receptor when affected and non-affected animals were compared (Fig. 4I). It is important to highlight that ESR2 was not detected in the mononuclear cells present in the infiltrates adjacent to affected efferent ductules.

### Hormone levels

Estradiol ( $E_2$ ) levels were increased 95% in the epididymal region of roosters affected by epididymal lithiasis compared with those not affected, whereas plasma levels were decreased 30% in these animals (Fig. 5A and B). Conversely, vitamin D3 concentrations presented a decrease of 86% in the epididymal region and a remarkable increase of about 11-fold in plasma of affected animals (Fig. 5C and D). Regarding testosterone levels, it was observed a drastic reduction in the concentration of this hormone within the epididymal region and plasma of roosters affected by epididymal lithiasis compared with non-affected animals (84 and 60%, respectively; Fig. 5E and F).

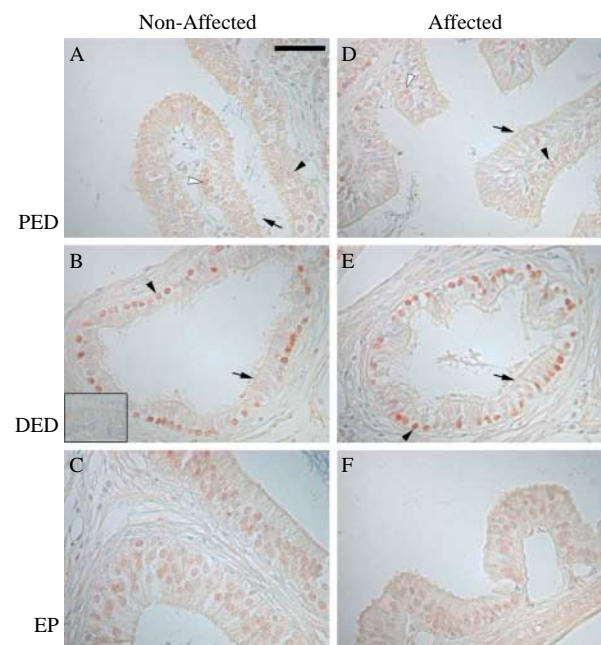
### Discussion

This study demonstrated that roosters affected by epididymal lithiasis had altered expression patterns of estrogen receptors ESR1 and ESR2 compared with control roosters. Paralleling these changes,  $E_2$  levels

were increased in the epididymal region, suggesting that alterations in the estrogen signaling may be associated with epididymal lithiasis. In addition, vitamin D3 and testosterone levels were decreased in the epididymal region of affected roosters. These results add to the limited data about epididymal lithiasis, pointing out that a local hormonal imbalance may be involved with the origin and progression of this anomaly, possibly by affecting the transepithelial calcium transport and/or fluid reabsorption in the efferent ductules.

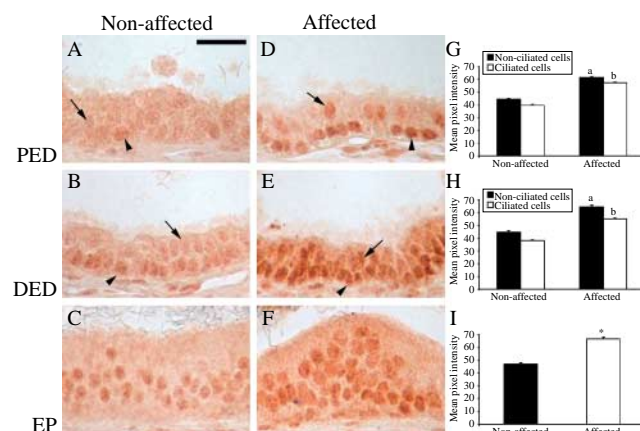
Roosters affected by the epididymal lithiasis presented decreased  $E_2$  and testosterone, but increased vitamin D3 plasma levels. It is difficult to determine the consequence of such alterations in calcium stone formation, but, corroborating our findings, altered hormonal profiles have been also associated with the development of calcium stones in the kidney of rodent and human (Iguchi *et al.* 1999, Yoshioka *et al.* 2010). In most cases, high levels of circulating estrogen prevents calcium crystal growth and aggregation (Iguchi *et al.* 1999, Yoshioka *et al.* 2010), whereas increased circulating vitamin D3 parallel a higher incidence of kidney stones (Worcester & Coe 2008, Shakhssalim *et al.* 2011). Because the circulating levels of hormones may not reflect exactly the physiology and function of specific organs, tissue concentrations of  $E_2$ , vitamin D3, and testosterone, were also investigated.

A remarkable characteristic of the avian efferent ductules is their participation in the reabsorption of a



**Figure 3** Immunodetection of ESR1 in the epididymal region of roosters not affected (A–C) and affected (D–F) by epididymal lithiasis. PED, proximal efferent ductule; DED, distal efferent ductule; EP, epididymal duct; white arrowheads, ESR1 positive non-ciliated cells; black arrowheads, ESR1 negative non-ciliated cells; arrows, ciliated cells; bar in A, 30  $\mu$ m; inset in B, negative control.





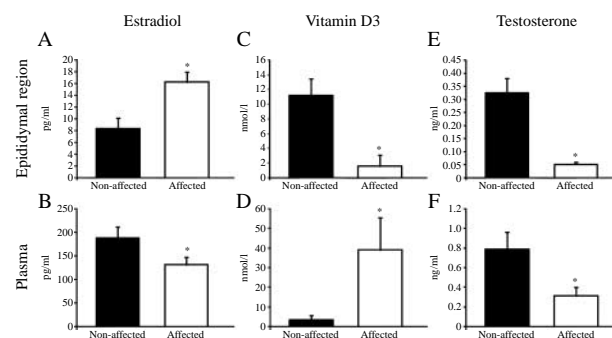
**Figure 4** Immunodetection of ESR2 in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (G–I) Graphical representation of the immunohistochemistry image analysis. PED, proximal efferent ductule; DED, distal efferent ductule; EP, epididymal duct; arrowheads: non-ciliated cells; arrows, ciliated cells.  $n=4$ ;  $a=P\leq 0.05$  between non-ciliated cells and  $b=P\leq 0.05$  between ciliated cells;  $*P\leq 0.05$ .

great amount of calcium, besides fluid reabsorption (Clulow & Jones 2004). Vitamin D3 is recognized as a key hormone in tissue calcium homeostasis (Cai *et al.* 1993, Hoenderop *et al.* 2001, 2005, Dick *et al.* 2003, Meyer *et al.* 2007). VDR is expressed within the mammalian male genital system (Stumpf *et al.* 1987, Schleicher *et al.* 1989, Johnson *et al.* 1996, Blomberg Jensen *et al.* 2010), playing an important role in sperm maturation (Blomberg Jensen *et al.* 2010). In birds, the efferent ductules exhibit greater amount of this receptor among the components of the epididymal region (Dornas *et al.* 2007). Because VDR was increased in the efferent ductules of roosters affected by epididymal lithiasis (Oliveira *et al.* 2008), the reduction in local vitamin D3 levels presently found suggests that the VDR upregulation was possibly a compensatory mechanism to reestablish local vitamin D3 action.

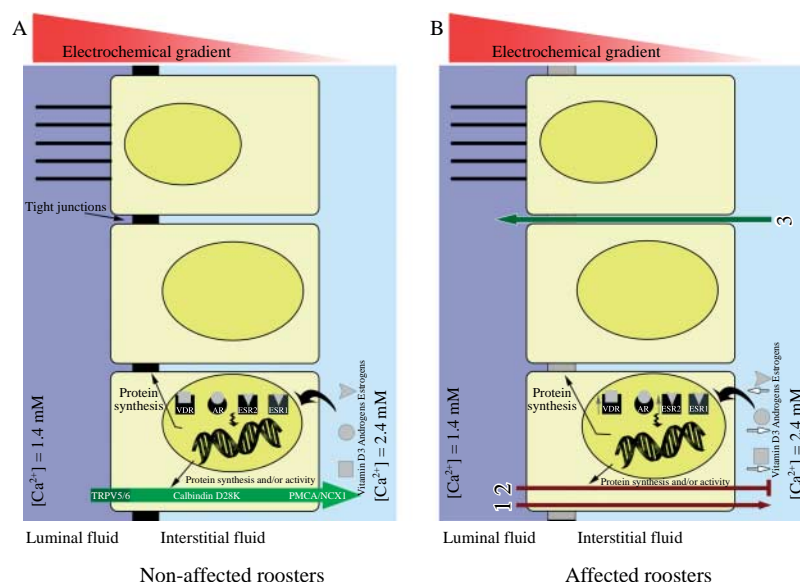
$E_2$  level was higher within the epididymal region of roosters with epididymal lithiasis than in non-affected animals. It is known that the biological function of  $E_2$  is mediated by estrogen receptors ESR1 and ESR2 (Carreau & Hess 2010), which shares structural similarities but also present functional differences (Kuiper *et al.* 1996, Heldring *et al.* 2007). Coincident with the  $E_2$  levels, ESR2 was also overexpressed in all segments within the epididymal region, whereas ESR1 levels were not affected. Involvement of ESR2 in other pathological conditions with soft-tissue calcification has been already demonstrated in other species, whereas ESR1 is commonly associated with a protective effect against calcification (Hodgin *et al.* 2001, Christian *et al.* 2006). It is known that estrogens are involved in regulation of the male tract luminal osmolarity and acidification (Joseph *et al.* 2010a, 2010b). Interestingly, acidification of luminal fluid inhibits calcium transcellular

reabsorption in the kidney of mammals (Bindels *et al.* 1994, Hoenderop *et al.* 2005, Topala *et al.* 2007). Therefore, it is possible that the impairment in estrogen responsive system within the epididymal region of roosters affected by epididymal lithiasis may also result in alterations in the luminal fluid pH with consequent negative effects in the local calcium homeostasis.

Vitamin D3,  $E_2$ , and testosterone play important roles in the maintenance of local calcium homeostasis as these hormones modulate the expression of key proteins involved in the transepithelial calcium transport. High levels of vitamin D3 and  $E_2$  are associated with increased calcium reabsorption, as they promote the expression of the transient receptor potential vanilloid channels (TRPV5), calbindin-D28k (CaBP-D28K), and plasma membrane calcium ATPase (PMCA), including in birds (Cai *et al.* 1993, Hoenderop *et al.* 2001, 2005, Van Abel *et al.* 2002, Dick *et al.* 2003, Kip & Strehler 2004, Oz *et al.* 2007). These proteins participate on the entry of calcium within the cell, its diffusion throughout the cell cytoplasm to the basolateral membranes and calcium extrusion to the extracellular environment respectively (Hoenderop *et al.* 2005). Moreover, low levels of testosterone increase the expression of the TRPV5 and CaBP-D28k (Hsu *et al.* 2010). Furthermore, besides the transcellular pathway, vitamin D3,  $E_2$ , and testosterone may also influence the paracellular calcium transport (Wada-Hiraike *et al.* 2006, Fujita *et al.* 2008, Kong *et al.* 2008, Braniste *et al.* 2009, Park *et al.* 2011). Therefore, even though  $E_2$  and testosterone are not considered as calciotropic factors, they may also affect the epithelial calcium transport both through the transcellular and paracellular pathways. Based on this information, and considering that the luminal stones consist primarily by calcium (Janssen *et al.* 2000, Mahecha *et al.* 2002), we hypothesize that the imbalance in vitamin D3, estrogen, and androgen responsive systems may result in calcium transport alterations through the transcellular and/or paracellular pathways (Fig. 6). Future detailed studies on the expression of key proteins involved in epithelial calcium transport should better clarify the mechanism of calcium handling in the epididymal region of roosters.



**Figure 5** Hormone levels of (A and B)  $E_2$ , (C and D) vitamin D3, and (E and F) testosterone in the epididymal region and plasma of roosters non-affected and affected by epididymal lithiasis.  $n=5$ ;  $*P\leq 0.05$ .



**Figure 6** Proposed mechanism for luminal calcium stone formation in the efferent ductules of roosters. (A) In roosters non-affected by epididymal lithiasis, calcium is reabsorbed against an electrochemical gradient (Clulow & Jones 2004) possibly by the transcellular pathway. This process is mediated by different key proteins (TRPV5 or TRPV6, calbindin-D28K as well as PMCA and NCX1) and regulated by vitamin D3, estrogens, androgens, and their receptors VDR, ESR1/ESR2, and AR (Hoenderop *et al.* 2005, Hsu *et al.* 2010). (B) In affected roosters, it is possible that the imbalance of VDR (Oliveira *et al.* 2008) and ESR2 levels as well as the local concentrations of vitamin D3, E<sub>2</sub>, and testosterone culminate in reduced (1) or impairment (2) in the transepithelial calcium transport. Also, the hormonal imbalance could interfere with paracellular calcium transport, resulting in the transport of this ion toward the lumen (3). As a consequence of events 1–3, individually or synergistically, calcium concentration within the lumen of efferent ductules would be increased creating a favorable microenvironment for calcium aggregation.

In conclusion, vitamin D3, E<sub>2</sub>, and testosterone levels as well as ESR2, but not ESR1, expression were altered in the epididymal region of roosters affected by epididymal lithiasis. These findings point out that alteration in these hormone responsive systems may result in disruption of local calcium homeostasis, which would culminate in the formation of a favorable microenvironment for the aggregation and development of the luminal calcium stones.

## Materials and Methods

### Animals and tissue preparation

The investigation was performed on the epididymal region of 18 adult cross breed roosters (*Gallus domesticus*) ~1–2 years old obtained from commercial sources and housed at the Universidade Federal de Minas Gerais facilities. The animals were kept under natural conditions of light, humidity, and temperature and were allowed free access to water and food (Socil III Guyomarc, Belo Horizonte, Brazil). The principles of research involving animals followed those advocated by the local Ethics Committee published by the CETEA/UFMG (<http://www.ufmg.br/bioetica/coep>).

After weighting, the roosters were anesthetized (i.p. lethal dose of sodium pentobarbital 50 mg/kg body weight) and blood samples were collected by cardiac puncture. The plasma was separated by centrifugation and stored at  $-20^{\circ}\text{C}$  for subsequent hormone measurements. Animals were then perfused intracardially with saline and 10% neutral buffered formalin (NBF) for immunohistochemical studies. After

fixation, the epididymal regions were dissected out from the testis. Alternatively, animals were perfused with saline solution only and after dissection; tissues were frozen in liquid nitrogen for western blotting and ELISA analysis.

### Diagnostics of epididymal lithiasis

The macroscopic diagnostic of epididymal lithiasis was performed by the tissue clearing methodology as previously described and validated (Mahecha *et al.* 2002, Oliveira *et al.* 2008). Briefly, tissues were rinsed in PBS, transferred to 0.5% (w/v) potassium hydroxide and immersed in glycerin solutions (1:2, 1:1, and pure glycerin). The animals were classified as affected or non-affected according to the presence or absence of epididymal stones in the epididymal region. Macroscopical findings were validated by histological evaluations of fixed epididymal fragments that were stained with hematoxylin and eosin (H&E).

### Histology and morphometry

NBF fixed tissues were processed routinely for paraffin embedding and sectioned at 5  $\mu\text{m}$ . Then, sections were stained with H&E and used for histological studies and morphometrical analysis by classical methodology (Weibel 1969, Oliveira *et al.* 2007). The volumetric density (Vv%) of the rete testis, proximal and distal efferent ductules, connecting ducts and epididymal duct as well as the connective tissue in the epididymal region was analyzed using a grid of 400 points. All the points of the grid incident in the ducts of the region were

scored and the result for each duct was divided by the sum of all points scored to obtain the Vv% of the parameters analyzed. Because differentiation between the connecting and epididymal ducts is difficult (Aire & Soley 2000, Oliveira *et al.* 2007), they were considered together.

### Western blotting

For the western blotting assays, epididymal regions of non-affected and affected roosters ( $n=5$  per group) frozen in liquid nitrogen were used. Following total protein extraction, samples were subjected to continuous electrophoresis using 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and blocked with 10% normal goat serum for 1 h at room temperature. After incubation for 1 h with rabbit anti-ESR1 (Clone 60C – Millipore, Temecula, CA, USA) or mouse anti-ESR2 (NCL-ER $\beta$  – Novocastra Laboratories, New Castle, UK) antibodies, used at 1:300 and 1:150 dilution, respectively, the blots were washed in PBS Tween 0.05% and then incubated in a biotinylated secondary antibody (Dako, Glostrup, Denmark) goat anti-rabbit (for ESR1) or goat anti-mouse (for ESR2) at 1:1000. The membranes were then incubated with the avidin–biotin complex (Vectastain Elite ABC Kit – Vector Laboratories, Burlingame, CA, USA) for 30 min and the immunolabeling was visualized with a solution of 0.1% (w/v) 3,3'-diaminobenzidine in PBS containing 0.05 chloronaphthol (w/v), 16.6 methanol (v/v), and 0.04% (v/v) H<sub>2</sub>O<sub>2</sub>. The quantification of ESR1 and ESR2 positive bands were estimated as described previously (Oliveira *et al.* 2008).

### Immunohistochemistry

NBF fixed, paraffin embedded fragments of the epididymal region of non-affected and affected animals ( $n=4$  per group) were used for immunohistochemical studies. To allow comparison between animals, the sections were run in parallel, and the staining was performed in two different sets to confirm the results. Sections were deparaffinized, rehydrated, blocked for endogenous peroxidase and submitted to antigen retrieval by a standard microwave method. The avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories). Sections were then incubated in 10% normal goat serum to block non-specific binding and then with the primary rabbit anti-ESR1 (Clone 60C – Millipore) or mouse anti-ESR2 (NCL-ER $\beta$  – Novocastra Laboratories) antibodies at the dilution of 1:50 and 1:25 respectively. The use of these antibodies has already being validated for rooster tissues (Oliveira *et al.* 2011). After several washes in PBS, the sections were exposed to a biotinylated goat anti-rabbit (for ESR1) or goat anti-mouse (for ESR2) secondary antibody (Dako) used at 1:50 dilution. The negative controls were obtained by the omission of the primary antibodies followed by the incubation with goat anti-rabbit IgG and goat anti-mouse IgG diluted 1:50 for ESR1 and ESR2 respectively. The sections were then incubated in the avidin–biotin complex (Vectastain Elite ABC Kit – Vector Laboratories). Finally, the immunoreaction was developed in 0.05% 3,3'-diaminobenzidine containing 0.01% (v/v) H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris–HCl buffer, pH 7.6 and stopped in distilled water. Sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

### Semi-quantitative immunohistochemical studies

The intensity of ESR1 and ESR2 immunostaining was estimated by computer-assisted analysis, based on previously reported protocols (Oliveira *et al.* 2008). Digital images from five different areas of the proximal and distal efferent ductules as well as the epididymal duct of each animal were taken by a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY, USA). The images were processed with Adobe Photoshop (Adobe Systems), converted to the grayscale mode and inverted. The images were then exported to Image-Tool Software (version 3.00, University of Texas Health Sciences Center, USA), for quantitative analysis. For this purpose, 25 nuclei of each cell type positive for the proteins investigated in the efferent ductules and epididymal duct were traced, measured 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.

### Hormone measurements

Vitamin D3, E<sub>2</sub>, and testosterone levels were dosed in the epididymal region and plasma by ELISA assays (Akhlaghi & Zamiri 2007, Ruwaan *et al.* 2010). For tissue analysis, epididymal region of affected and non-affected animals ( $n=5$ ) were frozen in liquid nitrogen and macerated in dry ice. Then, 100 mg tissue were suspended in 250  $\mu$ l of PBS (pH 7.4), homogenized and sonicated for 1 min. To optimize the results for E<sub>2</sub> and testosterone, lipid extraction enrichment with diethyl ether was performed according to a previous study (Hany *et al.* 1999). The enrichment of vitamin D3 in samples was performed by following manufacturer's instructions. The plasma used in the experiments was obtained after centrifugation of total blood (1800 *g* for 10 min) in heparin-coated tubes. ELISA measurements were performed according to the protocols provided by the manufacturers of the kits (E<sub>2</sub> and vitamin D3 – DRG Instruments GmbH, Marburg, Germany and testosterone Interkit – Bio Check, Foster City, CA, USA). It was used the same volume of samples per well of the ELISA plates. All samples were measured in duplicate within each assay and all the experiments were repeated in two independent assays. The intra- and inter-assay coefficients of variation (CVs) were 10 and 12% for vitamin D3, 4.6 and 7.8% for E<sub>2</sub>, and 7.4 and 5.2% for testosterone respectively. The sensitivity of the assays was 5.6 nmol/l, 9.7 pg/ml, and 0.083 ng/ml for vitamin D3, E<sub>2</sub>, and testosterone ELISA Kits respectively.

### Statistical analysis

Differences in hormone concentrations and ESR1 and ESR2 expression in the epididymal region of roosters non-affected and affected by epididymal lithiasis were statistically analyzed by the Student's *t*-test. The analysis of the morphometrical data (Vv%) was performed by using ANOVA and Newman–Keuls as *post hoc* for pairwise comparisons. Differences were considered significant at  $P \leq 0.05$ .



## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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