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Authors: N. Aknoun-Sail, Y. Zatra, I. Sahut-Barnola, A. Benmouloud, A. Kheddache, M. Khaldoun, S. Charallah, F. Khammar, A. Martinez, Z. Amirat

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N. Aknoun-Sail et al., Sex differences in Saharan gerbil β-catenin adrenal

N. Aknoun-Sail[1, 2], Y. Zatra[1, 2, 3], I. Sahut-Barnola[6], A. Benmouloud[1, 2, 4], A. Kheddache[1, 2, 5], M. Khaldoun[1, 2], S. Charallah[1, 2], F. Khammar[1, 2], A. Martinez[6], Z. Amirat[1, 2]

[1]Arid Zones Research Laboratory (LRZA), Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), Algiers, Algeria
[2]Faculty of Sciences, University of Algiers I Benyoucef Benkhedda, Algiers, Algeria
[3]Nature and Life Sciences Faculty, Saad Dahlab University of Blida (USDB 1), Blida, Algeria
[4]Department of Biology, Faculty of Sciences, M'Hamed Bougara University of Boumerdes (UMBB), Algeria
[5]Department of Biological and Agricultural, University Mouloud Mammeri Tizi Ouzou (UMMTO), Tizi Ouzou, Algeria
[6]Génétique, Reproduction et Développement (GReD), Centre National de La Recherche Scientifique CNRS, Institut National de La Santé & de La Recherche Médicale (INSERM), Université Clermont-Auvergne (UCA), France

Address for correspondence: Dr. N. Aknoun-Sail, Arid Zones Research Laboratory (LRZA), Faculty of Biology, University of Sciences and Technology Houari Boumediene (USTHB), BP 32, 16111, Algiers, Algeria, tel: 00 213 560 900451, fax: 00 213 21 24 72 17, e-mail: naouel.aknoun_fsb@usthb.edu.dz

ABSTRACT

Background: The adrenal cortex provides adequate steroidogenic responses to environmental changes. However, in desert rodents, the adrenocortical activity varies according to several factors especially sex, age, and seasonal variations. Herein, we examined the sex differences in the adrenal cortex activity and explored the involvement of sex hormones in the regulation of this function in Libyan jird *Meriones libycus*.

Materials and methods: Twenty-four adults male and female animals weighing 109-110g were captured in the breeding season and equally assigned into control and gonadectomised groups. Animal euthanasia was performed 50 days after the gonadectomy. Adrenal gland was processed for structural and immunohistochemistry study of β-catenin, whereas plasma was used for cortisol assay.

Results: The results showed that female adrenal gland weight was heavier than male and gonadectomy reduced this dimorphism. The adrenal cortex thickness was greater in the female than in the male, mainly due to significant development of the zona fasciculata. Females presented higher cell density in fasciculata and reticularis zones. The plasma cortisol was higher in females than in males. The immunolocalization of β-catenin showed that the expression was particularly glomerular in both sexes. However, in the female, the immunostaining was present in the zona reticularis while it was absent in the control male. Orchiectomy reduced zona glomerulosa cell density and induced hypertrophy of zona reticularis characterized by strong β-catenin immunoreactivity. However, ovariectomy leads to hyperplastic expansion and high β-catenin expression in the zona glomerulosa associated with zona fasciculata and reticularis hypoplasia distinguished by weak β-catenin immunostaining.

Conclusions: Results indicated that sex hormones had a major role in the regulation of the Saharan gerbil's adrenal homeostasis by modulating β-catenin signaling. Androgens seem to inhibit the Wnt β-catenin pathway and estrogens are activators of the adrenal inner zones.

Key words: adrenal cortex, gonadectomy, sex differences, structure, β-catenin, *Meriones libycus*

INTRODUCTION

The adrenal cortex is an essential endocrine tissue that produces steroid hormones controlling numerous physiological parameters that maintain body homeostasis. It is organized into three distinct zones each controlled by numerous hormones and paracrine factors. Mineralocorticoids that are involved in blood pressure and hydroelectrolytic homeostasis control are produced in the outermost zona glomerulosa (ZG), and glucocorticoids that are important in stress and immune response, which are synthesized in the zona fasciculata (ZF) and androgens that induce adrenarche in primates are produced in the innermost zona reticularis (ZR) [7]. To achieve a rapid and adequate response to intrinsic and extrinsic factors, the adrenocortical cells have developed mechanisms to ensure proper functioning throughout life. Wnt signaling is an essential paracrine pathway involved in adrenocortical homeostasis, zonation, development, and regeneration [18]. β-catenin, the main intracellular effector of canonical Wnt signaling, is highly expressed in the ZG in mice which regulates both the proliferation and steroidogenic activity of ZG cells to maintain the progenitor cell population and activating CYP11B2 cells expression producing aldosterone eventually replenish the ZF [6, 8]. Moreover, the activity of this gland is sexually dimorphic and has been shown in several animal species [17, 19]. Androgens cause the disappearance of the X-zone in male mice [9] while it persists in females; this zone reappears after male gonadectomy [7]. Recent cellular and molecular studies on the determinism of this dimorphism reveal that sex hormones are directly involved in the homeostasis and the remodeling of the adrenal cortex [10]. Indeed, studies have shown that the renewal of adrenal gland tissues in female mice shows a renewal rate 6.3-fold higher than in males, especially in the outer ZF [12].

Desert rodents are important models for understanding the cellular and molecular mechanisms that are involved in the development and homeostasis of the adrenal gland due to their adaptation in the extreme conditions of the biotope, which requires increased steroidogenic activity in order to respond to the needs of the environmental conditions. Previous studies reveal that the environment plays a major role in the modulation of adrenal responses, indeed the activity of the cortex is correlated either positively or negatively with the season and reproductive cycle [2, 16]. Circulating sex hormones are also factors that control this steroidogenic and homeostatic function. In desert rodents, the zone reticularis produces androgens, likely to participate synergistically with glucocorticoids to provide an anabolic effect and contribute to reproductive success [22].

In order to elucidate the role of sex hormones in the regulation of adrenocortical homeostasis, we compared the structure of the adrenal cortex in male and female *Meriones libycus* captured during the breeding season and analyzed the effects of gonadectomy on the remodeling and homeostasis of the adrenal cortex by exploring the weight parameters, structural changes, and the identification, by immunohistochemistry of a main mediator of Wnt/β-catenin signaling.

MATERIALS AND METHODS

Animals and sample collection

Animal experiments were carried out according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA), following approval by the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research. The permits and ethical rules were achieved according to the Executive Decree n˚ 10–90 completing the Executive Decree n˚04–82 of the Algerian Government, establishing the terms and approval modalities of animal welfare in animal facilities. Furthermore, it was recently supported by the local university ethical committee “Algerian Association of Experimental Animal Sciences” AASEA (Agreement Number 45/DGLPAG/DVA.SDA.14).

Twenty-four adult male and female gerbils (*Meriones libycus*, Lichtenstein, 1823), weighing 109±3g, were captured early morning in the field in the region of Béni-Abbès (30◦07′N 2°10′W, altitude 492m) during the breeding season (February-March) [4]. The adult reproductive condition was evaluated according to body weight (80-140g) and genital status was assessed during the breeding season. They were housed in individual cages (50cm in length, 35cm in width, and 30cm in height) in a temperature-controlled room (20–22°C) and a light/dark cycle respecting the natural circadian L/D cycle (11/13 in February March) and were fed with barley, bread, dates, some carrots, and some vegetables. The animals were divided into four groups: male control (MC, n=6), orchiectomized (ORX, n=6), female control (FC, n=6), and ovariectomized (OVX= 6).

Orchiectomy and ovariectomy

Six male and six female jirds were gonadectomized bilaterally under anesthesia induced by intraperitoneal injection of hydrochloride ketamine (Ketalar, Pfizer, NY, Toronto, Canada, 10 mg/kg administered *i.p.*) and xylazine (Xylamax, Bimeda-MTC; 10 mg/kg *i.p.*). The animals were then held supervised until they woke up, for 50 days. At the end of the experiments, all animals were euthanized between 9:00 and 11:00 a.m. The testes, ovaries of control groups; the seminal vesicles, and uterine horns of all animals were removed, weighed, and conserved for further studies. Adrenal glands were quickly removed, cleaned from their surrounding fat, and weighed separately. Adrenal glands were fixed in 10% neutral buffered formalin solution for 24h for histological and immunohistochemical studies.

Histology

Adrenals gland were dehydrated through successive exposure to increasing concentrations of ethanol (70%, 95%, and 100%), cleaned in the toluene, and after 24h of impregnation in paraffin in an incubator at 60ºC, adrenals were embedded in paraffin. Samples were then sectioned at 5 µm using a Leitz 1512 rotatory microtome (Marshall Scientific, Hampton, VA, USA). The resulting slices were placed in Superfrost®glass slides (Thermo Scientifc, Menzel-Gläser, Brausschweig, Germany). Following rehydration in decreased concentrations of ethanol (100%, 95%, 70%), parts of the serial slices were stained with Masson's trichrome while the others were subjected to immunohistochemistry.

Morphometric study

A comparison of adrenal cortex zones was performed by serially sectioning the whole adrenal at 5 µm. To account for the shape of the adrenal and allow for consistent measurements, counts were performed on twenty slides randomly chosen in the middle of the gland of all jirds. Zone depth was measured from the boundaries of each zone with 4 measurements per slide. The cell density was measured in ten slides randomly chosen on the grid area of 1713μm^2. The zone depth and cell density were measured using ZEN Blue Software (ZEN 2.3 Blue edition Carl Zeiss Microscopy GmbH).

Hormone assays

Plasma cortisol level was analyzed by electro-chemiluminescence immunoassay ECLIA (Roche Diagnostics, Meylan, France), using an automated hormone analyzer Elecsys 1010. Intra-and inter-assay coefficients of variation CV were 1.3/1.6%.

Immunohistochemistry

To detect the presence of β-catenin in the adrenal tissue, immunohistochemistry was performed using specific primary monoclonal antibodies (mouse) on tissues embedded in paraffin, after unmasking with sodium citrate 10 mM, Tween 0.05%. They were then incubated overnight with β-catenin antibody (BD610153) used at 1/500 dilution. The primary antibodies were detected with Signal Stain Boost HRP-Polymer solution (#8114S or #8125P, Cell Signalling). The quantitative evaluation of the immuno-reactivity was performed using the ImageJ software (http://mirror.imagej.net/docs/examples/stained-sections/index.html). Quantification is based on a subtraction operation of the light intensity between the positive and negative signal areas. To make comparisons, an Image Type conversion to RGB mode stack, the saturation threshold, and the standardization of the measured area are required.

Statistical analysis

All numerical data are expressed as means ± standard error of the mean (S.E.M.). Data were normally distributed values were analyzed by a two-tailed unpaired t-test (if comparing two groups) or a one-way ANOVA with Tukey's post-hoc test (if comparing multiples groups and variables) and it was considered significant when $p<0.05$. Statistical analyses were performed by using GraphPad Prism (version 7; GraphPad Sofware Inc., San Diego, CA, USA).

RESULTS

Sex differences and effect of sex hormones on weight parameters

Data for weight parameters were summarized in Table 1. It can be seen that body mass does not show a significant gender difference even if it is non-significant lower in females (-9%, p=0.1). Gonadectomy caused no effect on body mass both in male and female jirds (-2%, p=0.7; -2%, p=0.8) respectively (Table 1).

However, the weight of the relative adrenal is significantly higher in female compared to male (+22%, p=0.004) and gonadectomy reduced this dimorphism (+1%, p=0.8) due to a little effect on adrenals weight in the male (+8%, p=0.07) and a potential regression in the female (-10%, p=0.06) (Table 1).

The relative weight of the seminal vesicles and uterine horns were used as a reference to confirm the sex hormone reduction following gonadectomy. Indeed, the vesicles weight drastically reduced 50 days after orchiectomy (-76% of relative vesicle weight in ORX *vs*

CM, p=0.04) when uterine horns were also reduced in ovariectomized but none significantly (Table 1).

Gender differences in the jird adrenal structure

The adrenal gland in the male and female jird has a structure like all other mammals, with an elongated shape allowing the distinction between two parts, an external peripheral, adrenal cortex, and a central internal area, adrenal medulla, bounded all around by thick connective tissue. The gland is surrounded by a connective capsule made up of collagen fibers, fibroblasts, and blood capillaries (Figure 1A&B). The adrenal cortex is subdivided into three zones oriented from the outside to the inside: zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) (Figure 1 A&B). Morphometric measurements of the adrenal cortex revealed that the female has a larger adrenal cortex with a higher depth than those of the male (16%, p = 0.0003) (Figure 2).

The ZG is the outermost and thinnest zone of the cortex and appears as cell clusters forming arcing cords separated from each other by connective tissue containing blood capillaries (Figure 3 A&G). The depth of glomerulosa shows non-significant gender changes (Figure 2 A&B) but the zona is covered with a well-defined connective capsule in the male with many cells of small size and elongated in shape while in the female (Figure 3 A&G), the cells number is reduced compared to the male (-16%, p = 0.02) (Figure 4A).

The ZF is the thickest area of the adrenal cortex (Figure 1A&B). The cells are organized in long, narrow parallel cords, perpendicular to the capsule and directed towards the medulla (Figure 3B&H). The cells appear less acidophilic than other adrenal cortex cells due to the lipid droplets within these cells also called spongiocytes. The comparison between the male and the female ZF shows that the depth of the zona is greater in the female (26%, p=0.001) (Figure 2) with the presence of a higher number of cells per area unit (Figure 4B).

The ZR is the innermost layer is separated from the medulla by a connective tissue that seems developed in *Meriones libycus* (Figure 1 A&B); it is thinner than the ZF and is formed by an irregular network of anastomosed cords and cell clusters separated by bulky capillaries (Figure 3 C&I). Although the ZR presents numerous small cells which are more abundant in female (Figure 4C), it remains similar in depth within genders (Figure 2D).

Gonadectomy alters the adrenal structure both in male and female jird

Histological sections of the adrenal gland in gonadectomized animals show significant remodeling and structural changes in the cortex (Figure 1 C&D). Indeed, castration in the male induces the development of the cortex (+9%, $p=0.05$) (Figure 2) associated with hypertrophy of ZR (+45%, $p<0.0001$) (Figure 1 A&C). In the ovariectomized female, the adrenal gland appears withered (Figure 1 B&D), and the cortex thickness decreases due to the decrease of the ZF and ZR (Figure 2 A&C&D).

The ZG displays a change in the structure and organization since it loses organization in rounded cords, especially in the male (Figure 3 A&D) with a decrease of cell density (-40%, $p<0.0001$) (Figure 4A), whereas in the female, ZG presents numerous smaller cells than those of the control (+25%, $p<0.0001$) (Figure 4A) (Figure 3G&J).

The ZF appears disorganized in the male after orchiectomy, the parallel cord appearance of the cells disappears, they become small and of various shapes separated by thin connective tissue tracts containing blood capillaries (Figure 3 B&E); depth and cell density measurements show no significant variations (Figure 2C&4B). After ovariectomy, this area is also disorganized, the arrangement of cells in parallel cords is no longer visible (Figure 3 H&K), and it undergoes a significant reduction in the depth (-16%; $p = 0.05$) (Figure 2C) without changes in cell density number (Figure 4B).

The ZR is significantly hypertrophied in the orchiectomized (Figure 1C) and infiltrated by connective tissue (Figure 3 C&F) with an increase in depth zona (Figure 2D) and a reduction of cell number per unit (Figure 4C) while it decreases in the female ovariectomized (Figure 3 I&L) both in depth and cell density (Figure 2D&4C).

Hormonal effects

The mean concentration of plasma cortisol was higher in the female control *vs* male control values (+139%, $p=0.002$), after 50 days of gonadectomy, plasma cortisol was significantly elevated in male gerbils and reduced in the female compared with control values (respectively +121%, $p=0.02$; -55%, $p=0.04$) (Figure 5).

Immunolocalization of β-catenin in the adrenal cortex of the Libyan jird

Semiquantitative evaluation of the adrenal β-catenin immunoreactivity was summarized in Table 2. β-catenin is present in the adrenal cortex both in male and female as well as after gonadectomy (Figure 6); however, its distribution varies in both sexes. It is immunolocalized particularly at the ZG in both male and female (Figure 6A&B) with less important staining as well as at the innermost zones, in particular, ZR in the female (Figure 6A&B), while in the male, the β-catenin is also found at the ZG but more intensively compared to the female and lack fully in the innermost zones of the adrenal cortex (Figure 6A&B).

Gonadectomy induces a marked change in the distribution of β-catenin in the adrenal cortex. Indeed, it becomes internal in the male after castration where it forms a weakly immunolabeled trail in the ZF which appears more intensive in the ZR (Figure 6A&C). In the ovariectomized female, β-catenin is more immunostained in the ZG with more numerous cell layers (Figure 6B&D); on the other hand, the distribution of β-catenin in the inner adrenal cortex zones is reduced and less intense than in the control (Table 2).

DISCUSSION

Our results clearly showed that the adrenal cortex is more active in female than in male of *Meriones libycus*, this has been seen in the weight, structural and hormonal level. In male, the ZG is more developed than in female with a higher number of cells per unit area suggesting a greater mitotic activity. However, in female, the innermost zones especially the ZF and ZR show a large thickness associated with many cells per unit area, suggesting a remarkable proliferative activity. This finding corroborates with the cortisol plasma level which is higher in female than in male.

Immunohistochemical analyzes of β-catenin showed that this protein is mainly immunolocalized at the ZG in both sexes. Previous work has shown that it is responsible for the acquisition of the identity of ZG by the Wnt4 ligand [14]. However, in females, this protein is also found in innermost zona of the cortex forming a centripetal immunostaining gradient that is not observed in controls males. This distribution of β-catenin in the inner zona suggests that the Wnt-β-catenin signaling pathway is also taken especially in the ZR probably participating in the zonation process, which once again explains the intense development of the cortex in the female compared to the male. Recent studies have also revealed the presence of a centripetal decreasing gradient of β-catenin regulated by other paracrine factors including zinc and ring finger 3(ZNRF3)[3].

The literature has reported the existence of sex differences in the structure and the function of the adrenal cortex, in rat models, the activity is in favor of the female [20]; however, it has been reported in the Hamster that the cortex is more active in male than in female [19, 11]. These differences are reported at several levels of adrenal function, in fact, the development of the adrenal glands is different since the fetal cortex or X-zone disappears in the male after puberty and persists in the female and does not disappear until the time of pregnancy [15]. The female cortex of the mouse is 6-fold more active due to a high proliferative activity especially in the outer ZF and the increased recruitment of GLI1+ capsular stem cells, resulting in their higher differentiated ability [12]. Another study also demonstrated that the sex-related gene was more abundantly expressed in female rats compared to male rats [25]. Furthermore, the hypothalamic-pituitary adrenal axis is more active in female than in male [13, 26].

Experiments of sex hormones deprivation confirmed the gender differences. Indeed, orchiectomy in *Meriones* led to a reduction in the number of glomerular cells as well as hypertrophy of the ZR; in the female, the ZG cells are more abundant and more β-catenin immunoreactive with hypoplasia of the innermost zones. These findings were supported by the distribution of β-catenin which is found in hypertrophied reticularis zona of the male and by the disappearance of immunostaining in the female which is shown in the control. On one hand, this suggests that the Wnt/-β-catenin pathway is necessarily involved in the process of adrenal cortex zonation, especially the recruitment and increased renewal of steroidogenic cells. On the other hand, this pathway is regulated by gonadal hormones with probably a stimulating action of estrogen and an inhibitory action of androgens on the inner areas. The sex hormones mediate pituitary adrenal axis responsiveness to stress since the estrogen increased ACTH and corticosterone secretion in rats [28] while the androgen decreased the CRH, ACTH, and corticosterone concentrations both in the laboratory animals [23, 27] and the non-laboratory animals such as *Psammomys obesus [5]*, *Meriones libycus* [1] and *Gerbillus tarabuli* [29]. Sex hormones also act on adrenal androgens with, in particular, a stimulatory effect of estrogens in humans on the secretion of DHEA [21] and an indirect inhibitory effect by inhibiting the expression of 3β-HSD in the mouse [24]. Few studies report the effects of gonadectomy on the distribution of β-catenin, however, it has been shown that testicular androgens increase WNT signaling that antagonizes PKA, leading to slower adrenocortical cell turnover and delayed phenotype whereas gonadectomy sensitizes males to hypercorticism and reticularis-like formation [7]. Investigations using immunoblotting of beta-catenin throughout the adrenal gland and the assay of androstenedione should provide more knowledge about the action of testicular androgens in modulating adrenal activity.

CONCLUSIONS

The female *Meriones* adrenal gland is more active than the one of the male, characterized by the significant development of the zona fasciculata and reticularis leading to high plasma cortisol. Sex hormones participate in the modulation of this activity; androgens inhibit the activity of the steroidogenic cells in the innermost zone whereas estrogens are stimulators reducing the pool of progenitor cells located in zona glomerulosa. These actions of sex hormones seem to occur via the regulation of the Wnt β-catenin pathway which appears involved in the zonation of the innermost zone of the adrenal. Androgens seem to inhibit the Wnt β-catenin pathway in the inner zones and estrogens are stimulators of the Wnt β-catenin pathway in the inner zones.

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Table 1.

Table 1 Characteristics of some weight parameters in male and female Libyan jird *Meriones libycus* during the breeding season. Sex-dependant difference and effect of gonadectomy. CM: control male, ORX: orchidectomized, CF: control female, OVX: Ovariectomized. Data is reported as mean±SEM, n=6 animals/group

*CF *vs* CM, *ORX *vs* CM. * p<0.05, ***p<0.001

Animal groups		**Body weight (g)**	**Adrenals (mg/100 BW)**			**Reproductive tracts (mg/100 BW)**	
			Right adrenal	**Left adrenal**	**Adrenals**	**Seminal Vesicle**	**Uterine horns**
Male	CM	106.77±4.71	12.99±0.31	13.58±0.55	26.57±0.39	397±62	/
	ORX	104.53±4.09	13.14±0.65	15.64±0.61	28.77±0.98	113±5***	/
Female	CF	97.70±2.26	15.42±1.01	16.99±0.66	32.41±1.15*	/	230±118
	OVX	99.53±4.25	13.47±0.61	15.60±0.73	29.07±1.26	/	215±34

Table 2.

Groups		β-Catenin immunoreactivity (%)		
		ZG	**ZF**	**ZR**
Male	CM	62.39±0.7	7.11±0.7	12.38±0.6
	ORX	40.93±0.9***	14.59±1.7**	46.21±2.1***
Female	CF	59.23±0.6	12.14±0.9*	37.38±0.6***
	OVX	57.11±1.2	10.77±0.3	14.71±0.8###

Table 2. Quantification of β-catenin immunoreactivity in the adrenal cortex of *Meriones libycus* male and female during the breeding season. Effect of gonadectomy.

*CF *vs* CM, *ORX *vs* CM. * $p<0.05$, ***$p<0.001$; CF *vs* OVX. # $p<0.05$, ###$p<0.001$

Figure 1. Gender differences in the structure of the adrenal gland in the Libyan jird *Meriones libycus* during breeding season and effect of gonadectomy. Scale bar: 50µm.

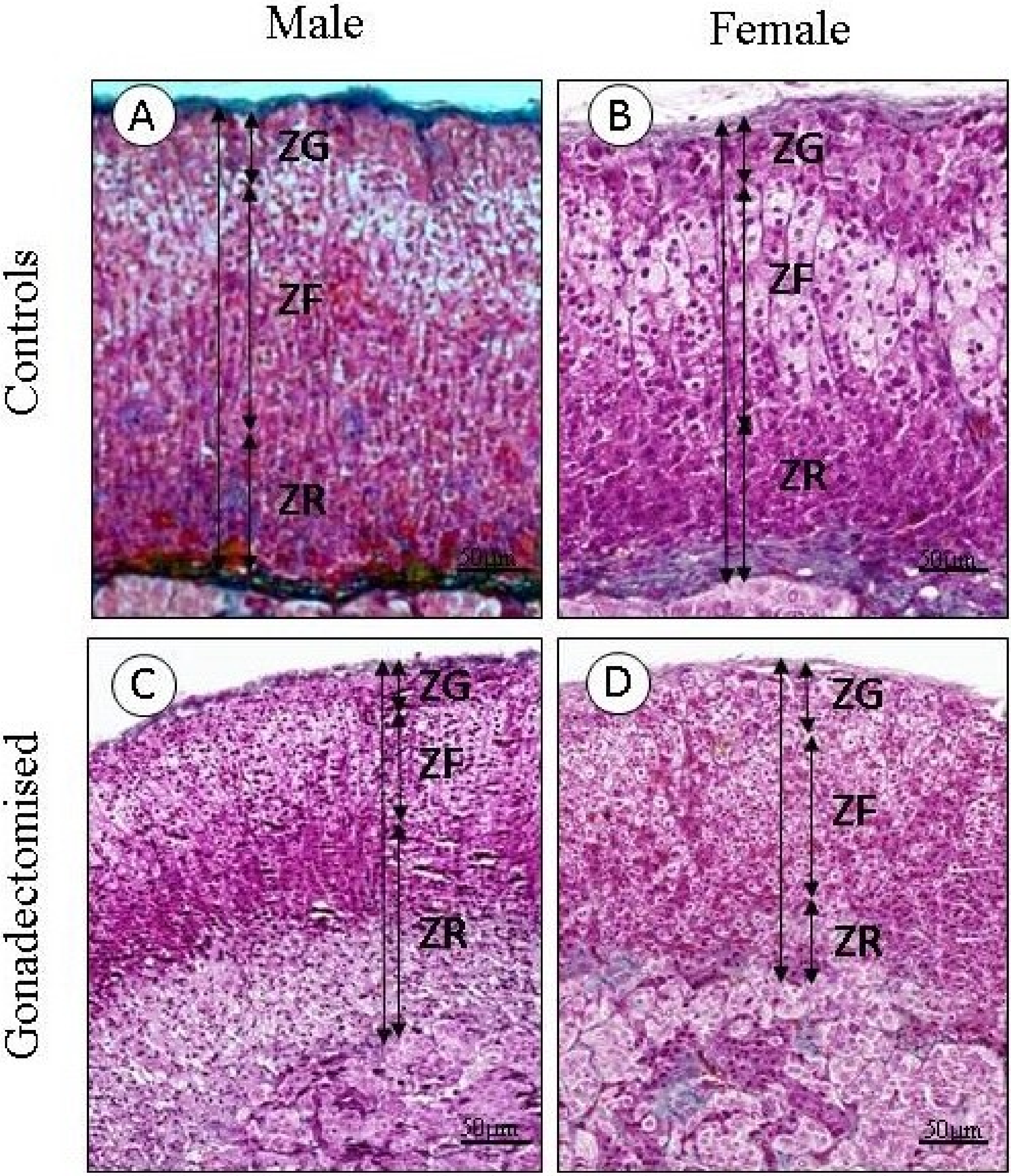
Figure 2. Gender differences and effect of gonadectomy on the morphometric measure of the depth of adrenocortical zone in *Meriones libycus* during the breeding season.

Figure 3. Gender differences in the structure of adrenocortical zone and effect of gonadectomy on this structure in *Meriones libycus* during breeding season. Scale bar: 20µm.

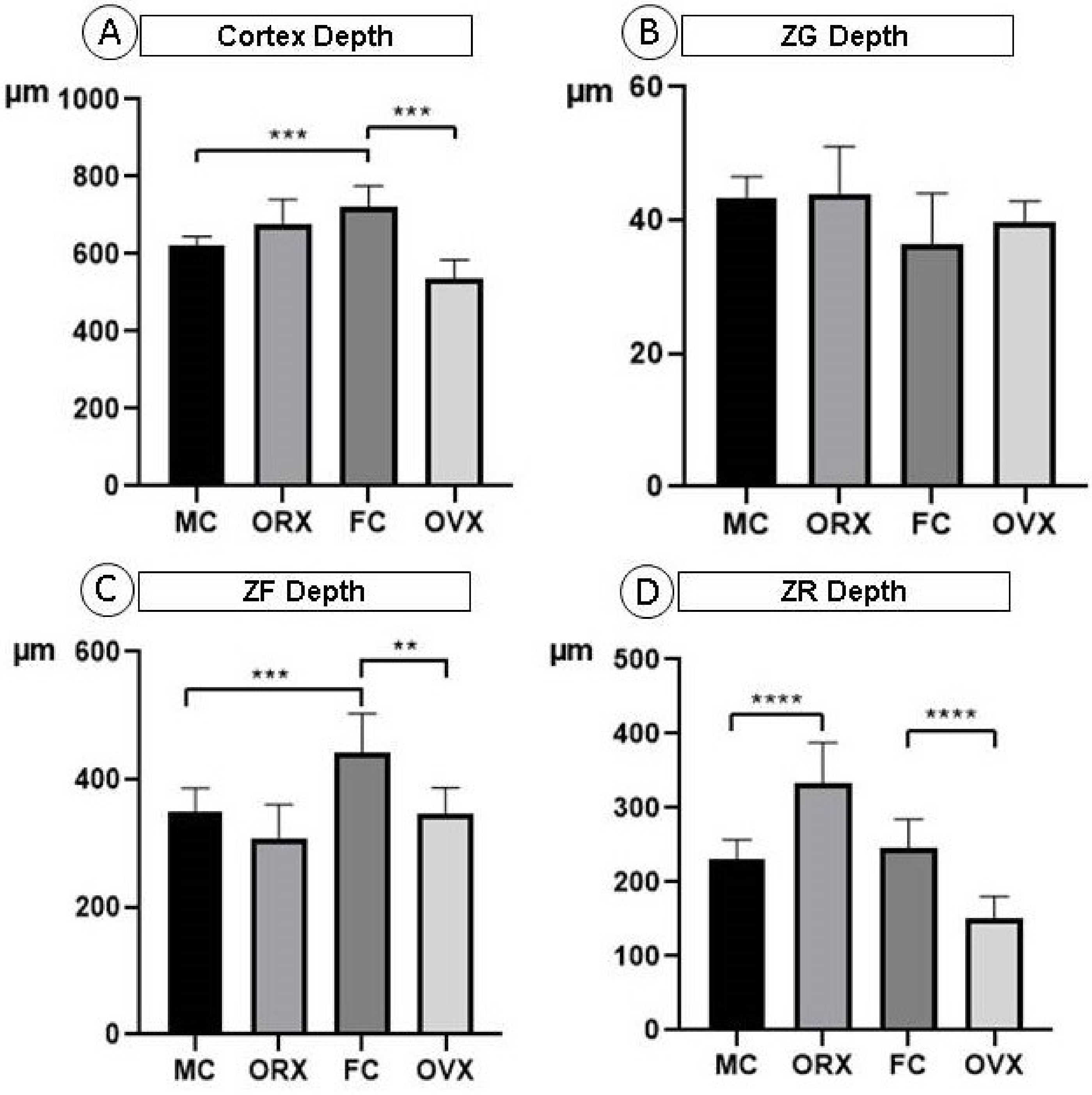
Figure 4. Gender differences in the cell density of the adrenocortical zone and the effect of gonadectomy in *Meriones libycus* during breeding season.

Figure 5. Sex differences in plasma cortisol concentrations and gonadectomy effects in male and female Meriones libycus during breeding season. Data is reported as mean±SEM, n=6 animals/group; **Control Female (CF) *vs* Control Male (CM), CF *vs* Gonadectomized female, $p<0.01$; *ORX *vs* CM. * $p<0.05$.

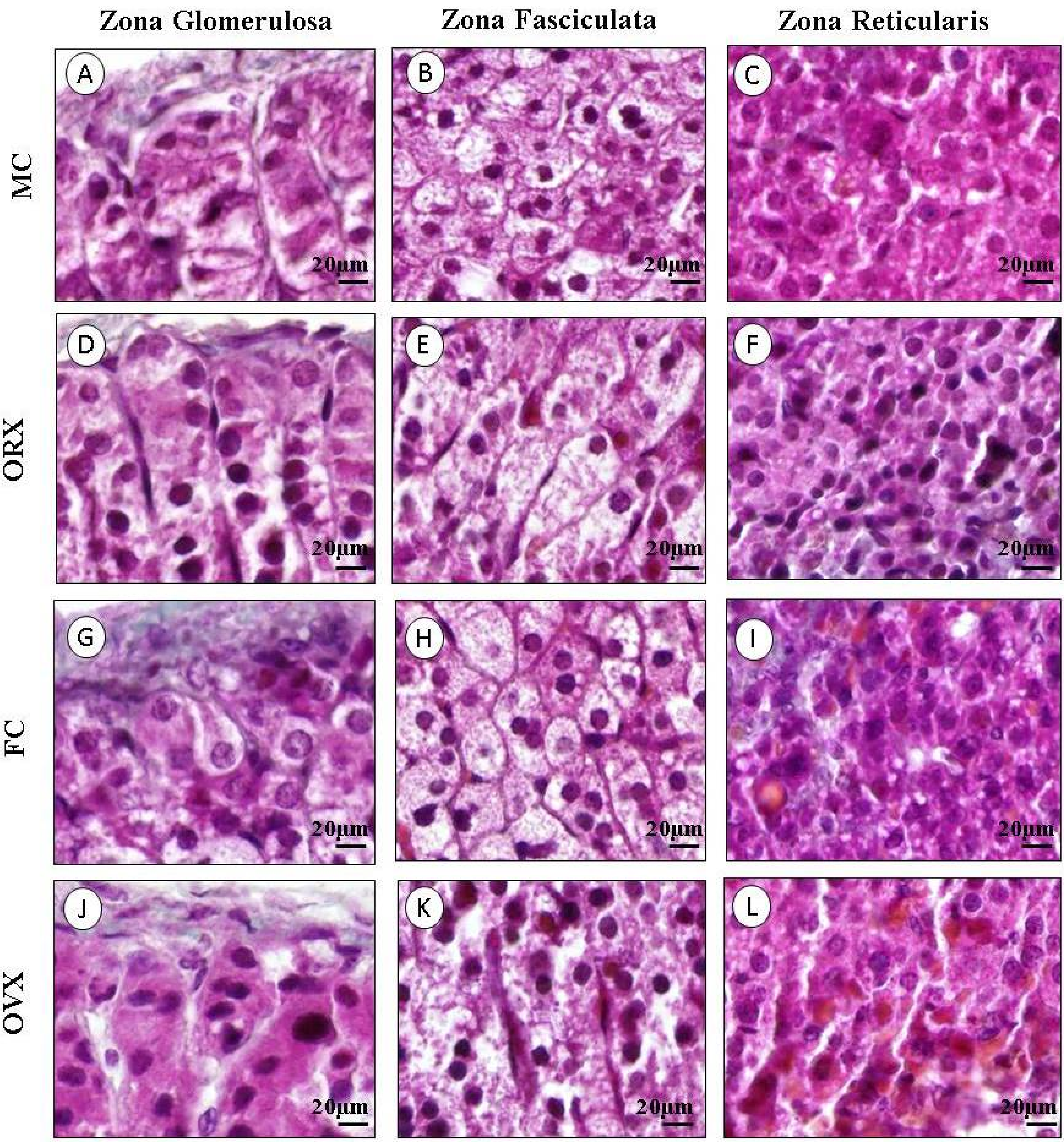
Figure 6. Immureactive β-catenin in the adrenal cortex of *Meriones libycus* male and female and the effect of gonadectomy.



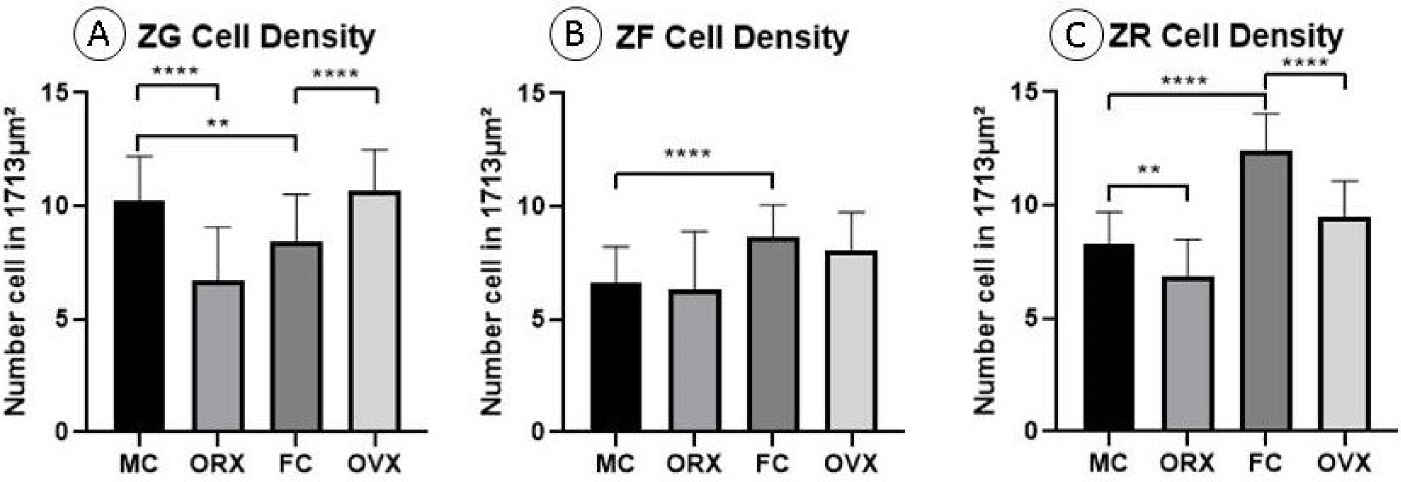
Male
Female
Controls
Gonadectomised
A
ZG
ZF
ZR
50μm
B
ZG
ZF
ZR
50μm
C
ZG
ZF
ZR
50μm
D
ZG
ZF
ZR
50μm



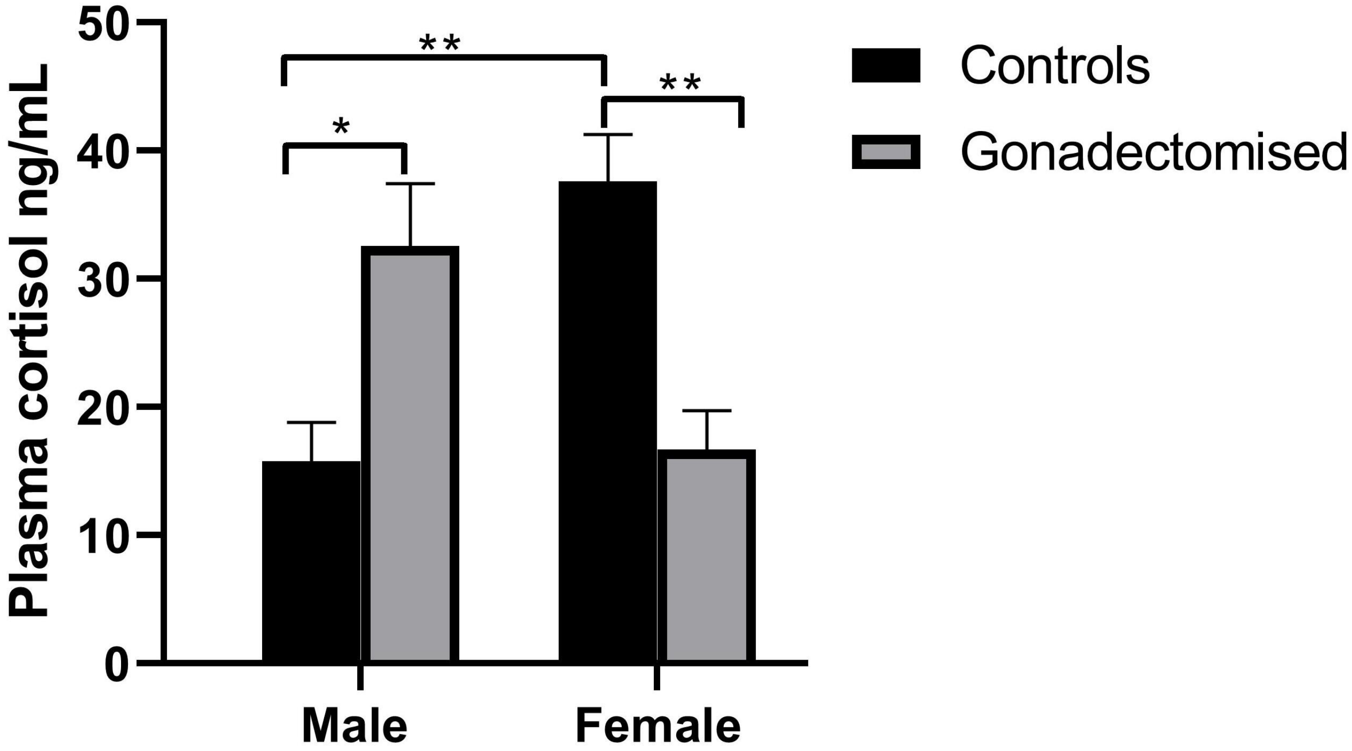
A
Cortex Depth
µm
1000
800
600
400
200
0
***
***
MC
ORX
FC
OVX
B
ZG Depth
µm
60
40
20
0
MC
ORX
FC
OVX
C
ZF Depth
µm
600
400
200
0
***
**
MC
ORX
FC
OVX
D
ZR Depth
µm
500
400
300
200
100
0
****
****
MC
ORX
FC
OVX



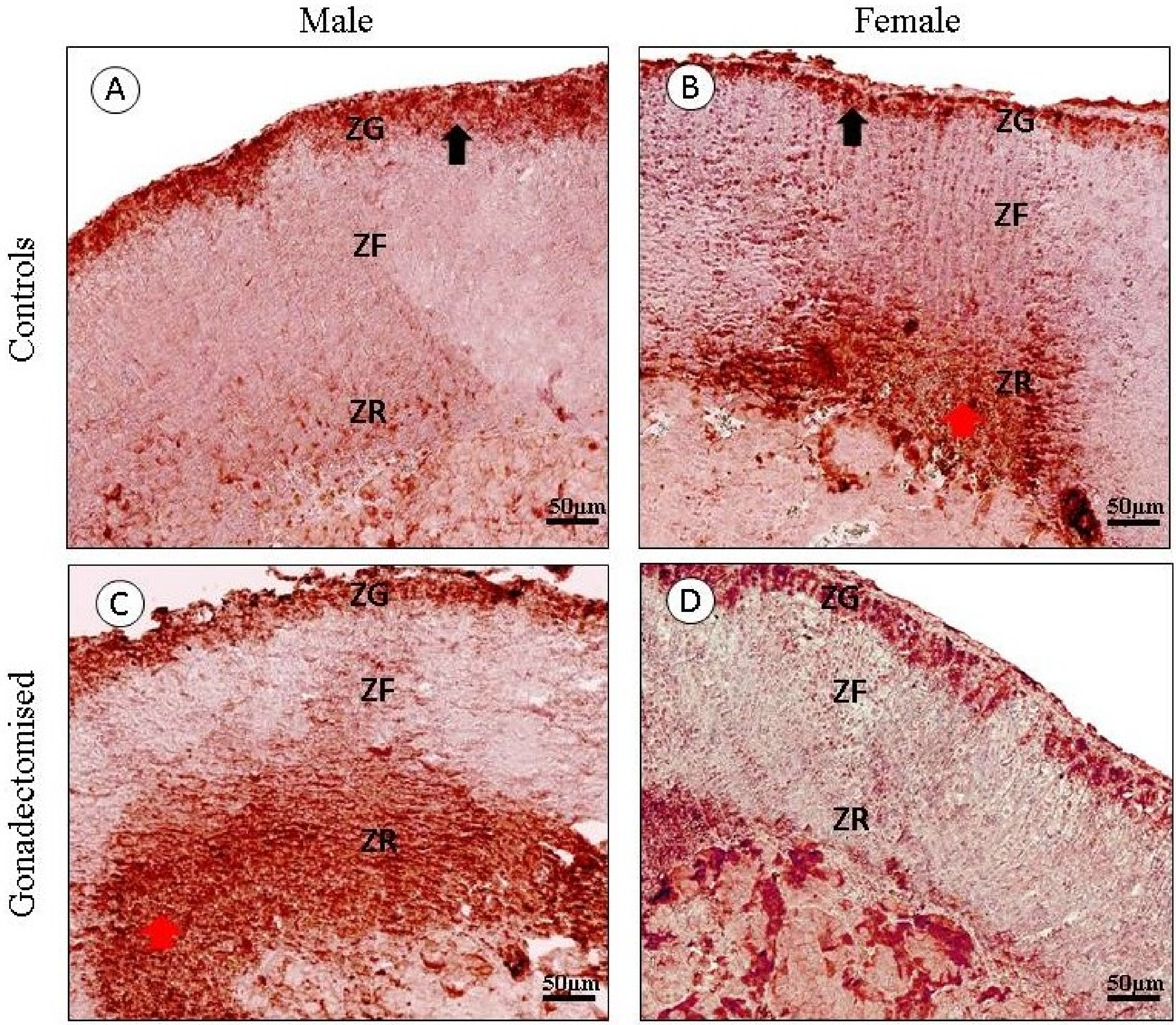
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
MC
ORX
FC
OVX
A
B
C
D
E
F
G
H
I
J
K
L
20μm



A ZG Cell Density
B ZF Cell Density
C ZR Cell Density
Number cell in 1713µm²
0
5
10
15
****
**
MC
ORX
FC
OVX



Plasma cortisol ng/mL
50
40
30
20
10
0
Male
Female
*
**
**
Controls
Gonadectomised



Male
Female
Controls
Gonadectomised
A
ZG
ZF
ZR
50µm
B
ZG
ZF
ZR
50µm
C
ZG
ZF
ZR
50µm
D
ZG
ZF
ZR
50µm