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Pituitary Adrenal Axis Activity in the Male Libyan Jird, *Meriones libycus*: Seasonal Effects and Androgen Mediated Regulation

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Many wild species exhibit seasonal cycles of testicular and adrenal functions suggesting important interrelationships between the two. However, data on desert rodents are very scarce. We investigated the pituitary adrenal (PA) axis activity of the Libyan jird from the Sahara desert, during the breeding and the non-breeding seasons. We explored, during the breeding season, the effects induced by orchidectomy and testosterone replacement (75 µg/40 µl sesame oil/100g BW). We found that the PA axis is more active during the non-breeding season and after orchidectomy with high ACTH and cortisol plasma concentrations ($P<0.05$). In the quiescent period, adrenal cells were hypertrophied in all cortical *zonae* ($P<0.05$), while orchidectomized jirds had thin *zona fasciculata* and thick *zona reticularis* with hypertrophied cells. Testosterone reduces the activity of the PA axis, probably by acting in the brain and/or pituitary. Here, we found that testosterone treatment led to a significant decrease in adrenocorticotropin levels together with structural remodeling of the adrenal cortex; analysis of androgen receptor (AR) densities in the adrenal cortex suggests that the AR mediates the direct effect of testosterone on adrenocortical morphology. This action may allow for homeostatic adjustments to occur, increasing individual fitness under extreme conditions in which this Saharan rodent species lives.

Key words: ACTH, cortisol, season, orchidectomy, testosterone replacement, Libyan jird.

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Environmental endocrinology has developed in order to better understand how hormones modulate and control physiological processes in animals exposed to extreme environmental conditions. The adrenocortical function plays a crucial role in response to physiological demand leading to the peripheral release of corticosteroids. Mineralocorticoids are involved in mineral homeostasis regulation while glucocorticoids, mainly controlled by adrenocorticotropic (ACTH) hormone, maintain energy balance but also increase in response to stress and could directly have an impact upon reproductive function by inhibiting GnRH release (BUBENIK *et al*. 1999). Elsewhere, recent studies have shown that the adrenocortical response can be controlled by various endocrine and paracrine factors which participate in remodeling and changing functional zonation, such as the transformation of glomerular cells into fascicular ones by subcapsular progenitor cells (PIHLAJOKI *et al*.

2015). The formation of ectopic gonadal-like tissue in the adrenal gland, due to the action of LH elevated in response to gonadectomy, can be viewed as an extreme example of adrenocortical remodeling leading to an increased androstenedione level (RÖHRIG *et al*. 2015).

Small mammals living in arid lands require permanent physiological adjustments ensuring both survival and successful reproduction. In arid habitats, rodents show seasonal reproductive activity only during the most favorable conditions (BELHOCINE *et al*. 2007; BOUFERMES *et al*. 2014; FUENTES *et al*. 1991; GERNIGON *et al*. 1994; KENAGY *et al*. 1999; KHAMMAR & BRUDIEUX 1984, 1987). Seasonal reproduction is generally correlated with adrenocortical activity as reported in the free-living degus *Octodon degus* (KENAGY *et al*. 1999), sand rat *Psammomys obesus* (AMIRAT *et al.* 1980; KHAMMAR & BRUDIEUX 1984), viscacha *Lagostomus maximus maximus* (RIBES *et al*. 1999) and South African striped mice *Rhabdomys pumilio* (SCHRADIN 2008). The variation of glucocorticoid levels during the breeding season (reviewed in ROMERO 2002) might influence gonadal androgens. Interestingly, injection of testosterone was found to reduce corticosterone levels in the free-living striped mice, *Rhabdomys pumilio*, a south African desert species (RAYNAUD *et al*. 2012) and to increase cortisol levels, while ACTH decreases in the Saharan sand rat (BENMOULOUD *et al*. 2014). These observations suggest functional relationships between the testes and the adrenal cortex. In fact, many previous studies have explored gonad-adrenal relationships in order to explain the X-zone disappearance in males after puberty (CRAMER & HORNING 1937) and sexual dimorphism affecting adrenal structure and function as reported in many small mammals (MALENDOWICZ & JACHIMOWICZ 1982; NIKICICZ *et al*. 1984). Moreover, previous studies have revealed a modulatory effect of sex steroids on pituitary adrenocortical response to ACTH under stress both for males and females in which androgens inhibit and estrogens stimulate this axis (HANDA *et al*. 1994a; VIAU & MEANEY 1996). Furthermore, AJDZANOVIC *et al*. (2015) reported that testosterone decreases the capacity for ACTH and corticosterone secretion in a rat model of andropause.

In the Libyan jird *Meriones libycus,* living in the Sahara desert, reproduction is characterized by a short breeding period, from the end of winter to the beginning of summer, followed by a long non-breeding season from June to the middle of winter (BELHOCINE *et al*. 2007; BOUFERMES *et al*. 2014). Cortisol was the predominantly produced glucocorticoid from the adrenals of this species, similar to reports in other desert rodents including *Meriones unguiculatus* (OLIVER & PERON 1964) and *Psammomys obesus* (AMIRAT *et al*. 1980). We found (unpublished personal data) 2-fold higher concentrations for cortisol (50 ng/mg) *vs* corticosterone (25 ng/mg) in adrenal tissue homogenate and 3-fold higher (28 ng/ml *vs* 8.7 ng/ml) at the circulating blood level. The aim of this study was to understand how testicular androgens can modulate the pituitary adrenal axis in response to the physiological needs of free-living *Meriones libycus*. We compared PA axis activity during the reproductive cycle and explored the influence of orchidectomy during the breeding season followed by testosterone replacement, upon the adrenal structure and morphometric changes occurring in the adrenal cortex, the immunolocalization of androgen receptors (ARs) in the adrenal cortical *zonae,* and the patterns of plasma ACTH and cortisol concentrations.

Material and Methods

Animals

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 in accordance 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 approved by the local university ethical committee "Association Algérienne des Sciences en Experimentation Animale" AASEA (Agreement Number 45/DGLPAG/DVA.SDA.14).

Meriones libycus, commonly named the Libyan jird, is a nocturnal, herbivorous and granivorous Saharan gerbil. It is of great interest because of its tolerance to water deprivation, thus providing an excellent model of adaptation to water restriction. Jirds live in superficial burrows arranged under large bushes of halophilic desert plants. They were trapped using Sherman traps in the Beni-Abbes area (30° 07′ N; 2° 10′ W, 497 m elevation) in the Algerian Sahara desert during the breeding season (February-March) and the middle of the non-breeding season (September-October). After trapping, animals were systematically identified according to the visual zoological criteria of PETTER (1961) and LE BERRE (1990); this desert rodent is characterized by a soft coat of light tawny color on the back, white on the belly. The tail is as long as the body, and ends with a bundle of dark brown hair. The plantar soles are covered with white hairs, and the nails are clear. The tympanic bullae

are hypertrophied. The captured females and juvenile males were systematically released near their burrows. Only adult male jirds were used in our study. They were weighed and housed in individual cages in a temperature-controlled room (20-22°C) with a light/dark regime representing the natural circadian L/D cycle (11/13 in February March; 13/11 in September October). They were fed with barley, bread, dates, some carrots, and vegetables. This diet furnishes all body requirements without the necessity of providing a source of drinking water in the laboratory. Daily control of their health status and hygienic maintenance was applied during the duration of the experiment.

Experimental design

All experiments began after one week of adaptation to captive conditions. Seven adult males were caught during the non-breeding season (nBC) and kept under conditions similar to that of breeding control. During the breeding season, 21 *M. libycus* individuals were divided into three groups: breeding control (BC, n=7), orchidectomized (ORX, n=7) and orchidectomized followed by testosterone replacement (ORX+T, n=7). Fourteen animals were orchidectomized bilaterally under anesthesia induced by intraperitoneal injections of ketamine hydrochloride (50 mg/kg, Ketalar, Pfizer, NY, USA). Spermatic arteries were ligated and the testes were rapidly removed. Muscular and cutaneous incisions were closed separately by silk sutures. Surgery wounds were disinfected and sprinkled with an antibiotic powder. The animals were then held under supervision until they awoke and then put in individual cages (50 cm in length, 35 cm in width and 30 cm in height), for 50 days, under daily control. After this period, seven of them underwent hormonal replacement treatment by subcutaneous injections of testosterone enanthate (75 µg/40 µl sesame oil/100g BW, Androtardyl, Bayer HealthCare) for seven days.

At the end of the experiments, all animals were euthanized under anesthesia between 9:00 and 11:00 a.m. The testes of control groups (nBC and BC) and the seminal vesicles of all animals were removed and weighed. Adrenal glands were quickly removed, cleaned from their surrounding fat and weighed. Right adrenal glands were fixed in 10% formalin solution for immunohistochemical studies, while left ones were stored at -20 ºC in 1 ml of phosphate buffered saline (pH 7.4). Blood was collected on ice, either in EDTA tubes for plasma ACTH assay or in lithium heparinized tubes for plasma cortisol assay. Biological samples were stored at -20 ºC until assays performed in the same series for each hormone.

Morphometric analysis

Adrenal glands were dehydrated in increasing concentrations of ethanol, cleared in butanol and embedded in paraffin. Sections of 5 µm thickness were stained by routine Masson's trichrome coloration. The specificity of the coloration was confirmed, and the sections were observed and photographed under a light microscope (Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera). Morphometric analysis was performed on six animals/experimental group using Axio vision software Rel. 4.6.3. Tissue thickness was measured under a 10x objective from twenty serial sections randomly chosen from the middle adrenal gland including medullary tissue. Eight measurements were taken around each adrenal section firstly for whole cortical thickness and then for each *zonae* thickness. Measurements were averaged for each animal. Cell and nuclear areas were measured in 100 randomly chosen cells for each *zonae* /adrenal section under a 100x objective.

Androgen receptor (AR) immunohistochemistry

Immunohistochemistry was performed by using the avidin-biotin complex method. Adrenal paraffin sections of 5 µm thickness were mounted on sterile water coated superfrost glass slides (Thermo Scientific, Menzel-Gläser, Braunschweig, Germany). Slide sections were deparaffinized in cyclohexane, rehydrated sequentially in 95% ethanol, 70% ethanol, distilled water and rinsed in 0.01 M phosphate buffered saline (PBS, pH 7.4). After antigen retrieval in citrate buffer (pH 6.0) using a pressure cooker as described in the kit prospectus (Vector Antigen Unmasking Solutions, Vector Laboratories, CA, H 3300), the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. In order to block non-specific site binding, the sections were washed in PBS pH 7.4 and then incubated with normal horse serum for 20 min at room temperature. The sections were incubated overnight at 4°C with primary antibody in a wet chamber. All antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal antibody (N20) raised against a peptide within the N-terminal domain of the human AR (sc-816), diluted at 1:300 in PBS, was used as the primary antibody. The slides were then washed in PBS and incubated with corresponding biotinylated secondary antibodies (Anti-Mouse IgG/Rabbit IgG; BA-1400, Vectastain Universal) for 1 h in a wet chamber. Visualization was performed by applying the ready to use Vectastain® Elite ABC Reagent at room temperature for 30 min. The binding sites of the primary antibody were visualized by using the chromogenic substrate

3,3'-diaminobenzidine-tetra-hydrochloride (SK-4100, DAB substrate kit for peroxidase) (DAB) and H_2O_2 solution for 1 min. Sections were counterstained with hematoxylin (Hematoxylin QS, H-3404; Vector Laboratories, Inc. Burlingame, CA 94010, USA) for 30 s before dehydration in a series of graded ethanol solutions, and cleared in cyclohexane. Images were captured by using a light microscope (Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera). Sections incubated with normal horse serum instead of primary antibody were used as negative controls. AR positive sections were defined as nuclear staining while cytoplasmic staining was non-specific. The number of AR-positive cells was counted and averaged by using Cell Target software, a bioinformatics application for morphometric analysis of cells (University of Alcala, Spain). Positively stained cells *vs*. a total number of 100 cells in each of the adrenal *zonae* were counted within a defined region of 45442.78 μm^2, under a x40 objective, from six animals/experimental group. The staining reaction was subdivided according to the percentage of stained nuclei as follows: Negative (-) = no nuclear immunostaining, weak staining (+) = less than 25% of positive nuclear staining, moderate staining (++) = 25-50% positive nuclear staining and strong staining (+++) = more than 50% of positive nuclear staining.

Plasma ACTH and cortisol assay

ACTH and cortisol assays were performed according to the electro-chemiluminescence immunoassay ECLIA (Roche Diagnostics, Meylan, France) protocol by using a Roche cobas e 411 analyzer for ACTH and an automated Elecsys 1010® hormone analyzer for cortisol. The intra-and inter-assay coefficients of variation for the ACTH assay were respectively 3.6 and 7.2 %, and the assay sensitivity was less than 1.0 pg/ml. The intra- and inter-assay coefficients of variation for the cortisol assay were, respectively, 2-3 % and 1.4-1.6 %. The lower limit of cortisol detection was 0.018 µg/dl. For the antibody derivate used in the kit of assay, the cross reactivities were 5.8% for corticosterone, 4.1% for 11-deoxycortisol, 1.50% for 17α-hydroxyprogesterone and less than 1% for all other tested compounds.

Statistical analysis

All numerical data are expressed as means ± standard error of the mean (S.E.M.). Data were normally distributed and obtained values were analyzed by one-way analysis of variance (ANOVA). The significance of differences between groups were verified using Tukey's test which was considered significant when $P<0.05$. Statistical analyses were performed by using GraphPad Prism v.5 software (GraphPad, San Diego, CA).

Results

Body mass and organs weight

Data for weight parameters are summarized in Table 1. Body mass, relative testes and vesicular weights were significantly lower during the non-breeding season *vs* breeding season (-19% and -46% respectively for BW and testes weight, $P<0.05$; -86%, $P<0.001$ for seminal vesicles weight). However, the relative adrenal weight didn't change

Table 1

Characteristics of some weight parameters in Libyan jird *Meriones libycus* during the reproductive cycle. Effect of orchidectomy and testosterone replacement. The body weight, testes and seminal vesicles were lower during non-breeding season while adrenal weight was higher. Orchidectomy caused no significant effect on adrenal weight; however, testosterone replacement increased it

Period of the year	Animal groups	Body weight (BW) (g)	mg/100g BW		
			Adrenals weight	Seminal Vesicles weight	Testes weight
Non-breeding season	nBC	88±2*	27.8±1.3	54±4***	833±28*
Breeding season	BC	108±8	25.7±1.9	394±68	1529±215
	ORX	106±4	25.4±1.9	92±14**	–
	ORX+T	93±3	30.6±1.6	378±25$^{\#\#}$	–

nBC: non-breeding control, BC: breeding control, ORX: orchidectomized, T: testosterone replacement.
Data is reported as mean±SEM, n=7 animals/group
*nBC *vs* BC, *ORX *vs* BC. * $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$
$^{\#}$ORX+T *vs* ORX. $^{\#\#}$ $P<0.01$.

significantly between the non-breeding and the breeding periods (+8% in nBC *vs* BC; P>0.05). The weight of the seminal vesicles was used as a reference in order to confirm androgen reduction together in the control non-breeding jirds and in the orchidectomized animals. Vesicle weight was drastically reduced 50 days after orchidectomy (-76% of relative vesicular weight in ORX *vs* BC, P<0.01) and it was fully restored after 7 days of testosterone replacement therapy (P<0.01) (Table 1). The orchidectomy didn't significantly influence body mass or adrenal weight although testosterone replacement caused a non-significant increase in adrenal weight (+20% *vs* ORX).

Histology of jird adrenal gland

In the intact jird, histological study showed that the adrenal cortex has a thick connective tissue capsule (Fig. 1A, 1B) and was divided into three zones as commonly described for all other mammals. It's arranged into three concentric *zonae*, the outermost *zona glomerulosa*, the middle *zona fasciculata* and the inner *zona reticularis* (Fig. 1B). The medulla was sharply demarcated from the cortex by a band of connective tissue which sends strands into the medulla and which is developed during the breeding season (Fig 1B). The *zona glomerulosa* occupied approximately 7-8 layers of vacuolated cells during the non-breeding season (Fig. 2A) and 4-5 layers during the breeding season (Fig. 2C). The cell cords of the *zona fasciculata* were closely packed. In the outer half of this *zona*, the cells were highly vacuolated mainly during the non-breeding season but larger than those of the *zona glomerulosa* (Fig. 2A). The vacuolization markedly decreased in the inner half of the *zona fasciculata*. The *zona reticularis,* formed of deeply stained cells arranged in irregular short rows, is narrow during the breeding season (Fig. 2D) and larger during the non-breeding season (Fig. 2B).

Orchidectomy induced cell disorganization in the outer *zonae* with disappearance of cell cords in the *zona fasciculata* and extreme vacuolization of glomerular and fasciculata cells (Fig. 2E). The *zona reticularis* showed numerous small-sized cells which were infiltrated with connective tissue cells with elongated nuclei (Fig. 2F). Testosterone replacement restores the changes affecting adrenal structure (Fig 2G, 2H).

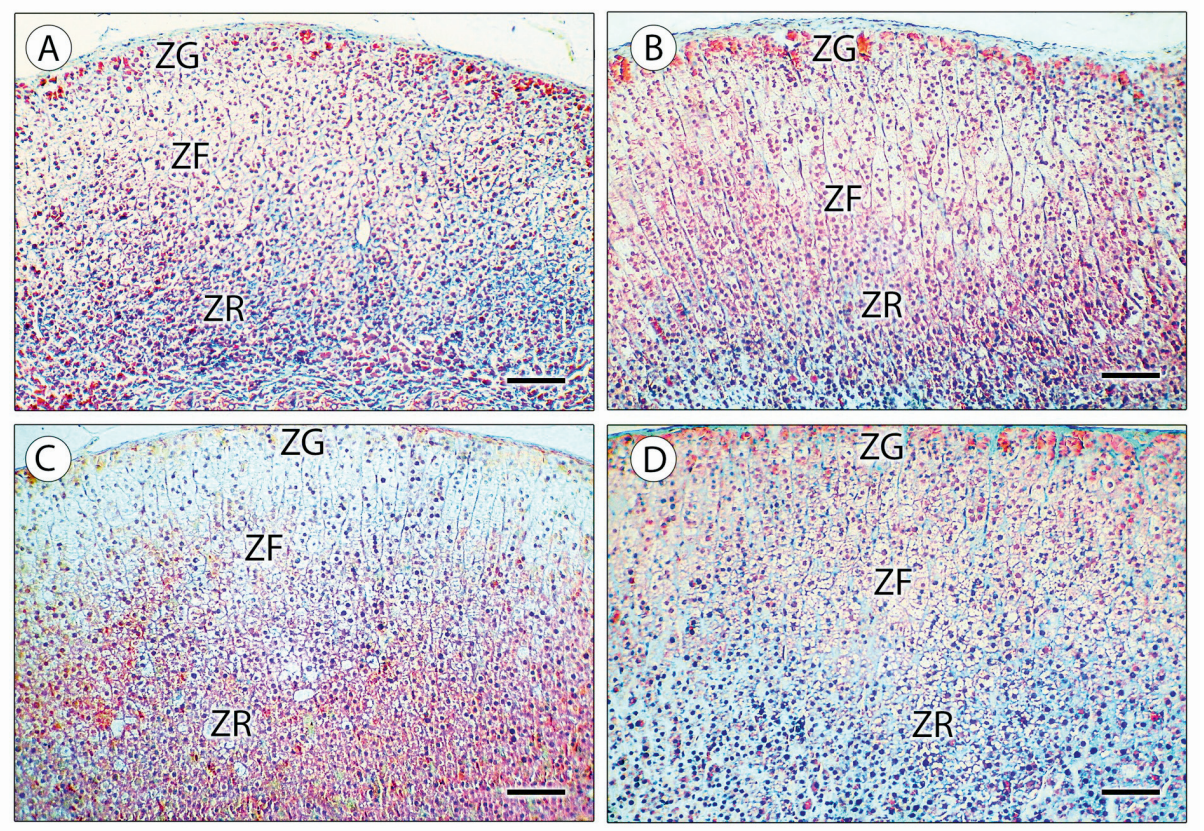

Fig. 1. Structure of the adrenal gland in the Libyan jird *Meriones libycus* during breeding and non-breeding seasons. Effect of orchidectomy and testosterone replacement. Orchidectomy weakly increased the adrenal cortex (C) with a hypertrophy of the *zona reticularis* and regression of *zona fasciculata*. Testosterone replacement causes a further increase in adrenal cortex height with restoration of *zona fasciculata* status.
A: nBC; B: BC, C: ORX, D: ORX+T. *ZG*: *zona glomerulosa*, *ZF*: *zona fasciculata*, *ZR*: *zona reticularis.* Scale bar: 100 = µm.

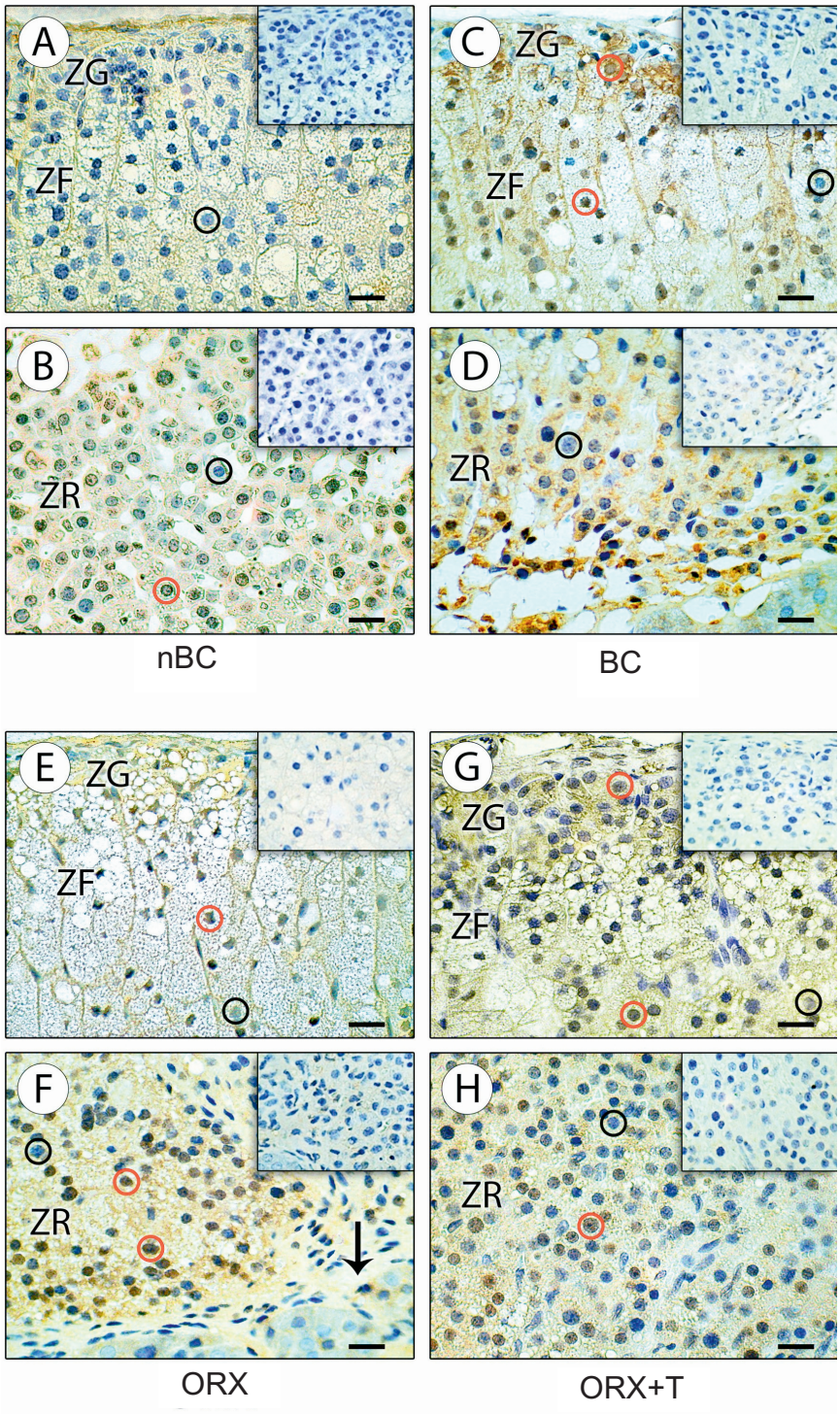

Fig. 2. Immunolocalization of androgen receptors (AR) in the adrenal cortex of *Meriones libycus* during breeding and non-breeding seasons.
Non-breeding control nBC (A, B), BC (C, D), ORX (E, F), ORX+T (G, H). *Zonae glomerulosa* and *fasciculata* were shown in figure 2 (A, C, E, G) and *zona reticularis* in figure 2 (B, D, F, H). Red circles indicate immunostained nuclei, black circles indicate negatively stained (hematoxylin) nuclei. Large numbers of AR positive cells were observed in all zonae in nuclei; orchidectomy intensified the immunopositive AR in nuclei of *zona reticularis* and reduced it in the *zona glomerulosa* and *fasciculata*. Testosterone caused important labeling in all cortical zonae. Scale bar = 20 µm.

Morphometric analysis of the adrenal cortex

Data for morphometric measurements are summarized in Table 2. During the non-breeding season, the adrenal gland (Fig. 1A) was characterized by an enlarged cortex (+9% *vs* BC, $P<0.01$) (Table 2) compared to the breeding season (Fig. 1B). The *zona glomerulosa* was significantly more developed (+26% *vs* breeding season, $P<0.05$) and the cell and nuclear areas were significantly higher in

all cortical zonae (respectively +14% and +12% in *zona glomerulosa*, P<0.01; +22%, P<0.001 and +6%, P<0.05 in *zona fasciculata* and +41%, P<0.001 and +17%, P<0.01 in *zona reticularis*) (Table 2).

Orchidectomy induced a slight increase in the adrenal cortex thickness (Fig. 1C) (+6%, P<0.05) due to a significant hypertrophy of the *zona reticularis* (+125%, P<0.001) associated with the regression of *zona fasciculata* (-31%, P<0.001) and a weak development of *zona glomerulosa* (+23%, P>0.05). Indeed, cell and nuclear areas were reduced after orchidectomy, respectively in the *zona glomerulosa* (-6%, -8% P<0.05) and especially in the *zona fasciculata* (-21%, -24%, P<0.001), but only cell area increased significantly in the *zona reticularis* (+30%, P<0.001) (Table 2).

Testosterone replacement induced an enlargement (Fig. 1D) of the adrenal cortex (+10%, P<0.01). It clearly restored the *zona fasciculata* morphometry (+66% in thickness, P<0.001 and +51% and +22% respectively in cell and nuclear areas, P<0.01) while no changes occurred in *zonae glomerulosa* and *reticularis* which showed a significant decrease in cell area (-13%, P<0.05) (Table 2).

Immunolocalization of ARs in the adrenal cortex of the Libyan jird

Semi-quantitative evaluation of the adrenal AR immunostaining is summarized in Table 3. Immunoreactive ARs were detected in all adrenal zonae although they were less abundant during the non-breeding season as well in the *zona glomerulosa*

Table 2

Morphometric study of the adrenal cortex in the Libyan jird *Meriones libycus* during the reproductive cycle. Effect of orchidectomy and testosterone replacement. Adrenal cortex was higher in non-breeding season with hypertrophied cells of all cortical layers. Orchidectomy weakly increased the adrenal cortex with significant hypertrophy of *zona reticularis* and regression of *zona fasciculata*, testosterone restores this effect. Cell and nuclear areas: orchidectomy reduced the cell status in *zona glomerulosa* and *zona fasciculata* when *reticularis* increased. Testosterone restores the effect

Groups	Tissue thickness (µm)				Cell and Nuclear areas (µm^2)					
	Total Cortex	ZG	ZF	ZR	ZG		ZF		ZR	
					CA	NA	CA	NA	CA	NA
nBC	1194±25**	110±3*	826±19	242±11	277±4**	94±1,3**	540±6***	125±1.2*	404±4***	117±1.3**
BC	1092±16	87±12	824±10	206±9	243±5	84± 1.7	444±8	118±2.4	286±4	100±1.4
ORX	1156±34*	107±4	567±20***	463±22***	227±3*	77±1.3**	351±6***	90±1.4**	366±6***	91±1.6**
ORX+T	1272±40$^{\#\#}$	99±2	944±27$^{\#\#\#}$	427±13	232±3	73±1.2	532±6$^{\#\#}$	110±1.6$^{\#\#}$	320±3$^{\#}$	94±1.2

BC: breeding control, nBC: non-breeding control, CA: cell area, NA: Nuclear area, ORX: orchidectomized, T: testosterone replacement. *ZG*: *zona glomerulosa*, *ZF*: *zona fasciculata*, *ZR*: *zona reticularis.*

* nBC *vs* BC, * ORX *vs* BC. * P<0.05, **P<0.01, ***P<0.001

$^{\#}$ ORX+T *vs* ORX. $^{\#}$P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001.

Data used 6 animals/group with 20 adrenal serial sections for tissue thickness and 100 cells/zonae for cell and nuclear areas

Table 3

Semi-quantitative assessment of AR immunostaining in the adrenal cortex of the Libyan jird *Meriones libycus* during the breeding and non-breeding seasons. The immunostaining was less abundant in non-breeding (nBC) than breeding season (BC). Orchidectomy (ORX) reduces it in *ZG* and *ZF* and increases it in *ZR*. Testosterone replacement (ORX+T) intensified immunostaining strongly in all cortical layers

Groups		*Zona glomerulosa*	*Zona fasciculata*	*Zona reticularis*
nBC	Non-breeding season	+	+	++
BC	Breeding season	++	+++	+
ORX		+	++	+++
ORX+T		+++	+++	+++

ZG: *zona glomerulosa*, *ZF*: *zona fasciculata*, *ZR*: *zona reticularis*.

The intensity of ARs positive stained cells is expressed as a percentage: (-) no staining; (+) weakly positive staining of <25% cells; (++) moderately positive staining of >25 % cells; (+++) strongly positive staining of >50 % cells; Data used 6 animals/ experimental group.

and *fasciculata* (Fig. 2A) than in the *zona reticularis* (Fig. 2B), compared to the breeding control (Fig. 2C, 2D) for which positive AR immunoreactivity was found in all adrenocortical *zonae*. However, the labeling was expressed moderately in the *zona glomerulosa* and strongly in the *zona fasciculata* (Fig. 2C), while the *zona reticularis* (Fig. 2D) was weakly immunostained.

Orchidectomy induced more immunostaining in the *zona reticularis* (Fig. 2F) and reduced it in the outer *zonae* (Fig. 2E) while testosterone replacement caused important recurrence of labeling (Table 3) in all cortical zonae (Fig. 2G, 2H).

ACTH and cortisol concentrations of the Libyan jird

In the Libyan jird, plasma ACTH and cortisol concentrations were about 5 folds higher ($P<0.05$) during the non-breeding than during the breeding season (Fig. 3) revealing important seasonal variations of the PA axis activity of this desert gerbil.

Orchidectomy performed on sexually active jirds also induced an increase in plasma concentrations of ACTH (148%, $P<0.001$) and cortisol (121%, $P<0.05$) while testosterone replacement drastically reduced (Fig. 3) plasma ACTH levels (-94%, $P<0.001$), whereas cortisol concentrations showed only a slight reduction (-26%, $P>0.05$) (Fig. 3).

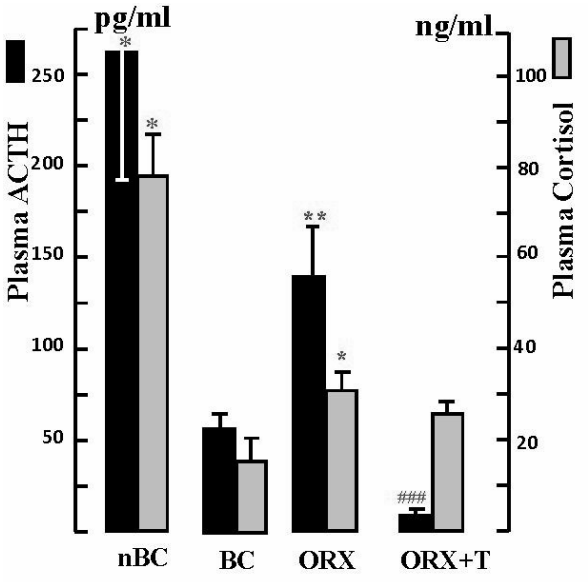

Fig. 3. Plasma concentrations of cortisol (ng/ml) and ACTH (pg/ml) during the reproductive cycle of *Meriones libycus*. Plasma ACTH and cortisol were higher during the non-breeding season. Orchidectomy raised plasma concentrations of both ACTH and cortisol. Testosterone replacement restores this effect.
nBC: non-breeding control, BC: breeding control, ORX: orchidectomy, T: testosterone replacement.
*: nBC *vs* BC; ORX *vs* BC, * $P<0.05$, ** $P<0.01$
#ORX+T *vs* ORX, ### $P<0.001$.
Data used 7 animals /group

Discussion

Previous papers have reported that the Libyan jird *Meriones libycus* is a seasonal breeder (BELHOCINE *et al*. 2007; BOUFERMES *et al*. 2014) with a short breeding period during spring. In this paper, we reported for the first time that during the non-breeding period (in autumn), the pituitary adrenal axis of the male of this desert rodent was more active than during the breeding season. Indeed, circulating ACTH as well as cortisol plasma concentrations were elevated when testicular activity was reduced. These results suggest negative relationships between male sexual hormones and pituitary adrenal axis. Therefore, we focused on how adrenal androgens could modulate the activity of the PA axis involved in the response to the physiological homeostatic needs of the male Libyan jird *Meriones libycus*, living in its natural Saharan environment.

Several papers have reported relationships between pituitary-adrenal axis activity and gonads (SEALE *et al*. 2004a). On the other hand, sexual differences in HPA axis activity are well established in several mammal species, such as sheep (CANNY *et al*. 1999) and rodents (HANDA *et al*. 1994a). These differences are partly due to circulating sex hormones (BURGESS & HANDA 1992; HANDA *et al*. 1994a). Our study shows an important role for the androgen testosterone in the suppression of adrenocortical activity in male jirds. Future studies should examine the role of female sex steroids on this parameter. Studies in laboratory rats reported that estrogens stimulate adrenocortical function and those females generally display higher PA axis activity. Unfortunately, in addition to the lack of information on female desert rodents, there is also very little data that refers to how rodents living in arid environments respond to environmental stress.

Our results are at odds with previous studies in species showing seasonal glucocorticoid variation similar to that of androgens, in order to accommodate and maintain energy for reproduction as reported in the sand rat *Psammomys obesus* (AMIRAT *et al*. 1980; KHAMMAR & BRUDIEUX 1984), in the male red deer *Cervus elaphus* (HUBER *et al*. 2003) and the viscacha *Lagostomus maximus maximus* (FILIPPA & MOHAMED 2006; RIBES *et al*. 1999). Nonetheless, our results agree with those found in the European beaver *Castor fiber L* (CZERWINSKA *et al*. 2015), free living degus *Octodon degus* (KENAGY *et al*. 1999), the nocturnal garden dormouse (BOULOUARD 1971) and the male South African striped mouse *Rhabdomys pumilio* (SCHRADIN 2008).

During the non-breeding period, the increase in adrenocortical thickness was due, in part, to cell

hypertrophy in the three *zonae*, while plasma ACTH and cortisol concentrations were enhanced. This observation is probably related to the need of glucocorticoid and mineralocorticoid hormones in order to maintain adequate homeostasis under unfavorable environmental conditions (outside of the breeding season).

On the other hand, during the breeding season, orchidectomy induced regression of *zona fasciculata* thickness due to cell area decrease, probably associated with apoptotic cells, as reported in *Psammomys obesus* living in the same desert area (BENMOULOUD *et al*. 2014) while the *zona reticularis* became hypertrophied. In future studies, TUNEL assays and BrDU labeling could confirm if the morphometric changes occurring in the adrenal cortex *zonae* result from a decrease in apoptosis and/or the cell proliferation increase, as observed in the Androgen Receptor Knock out (ARKo) mice (MIYAMOTO *et al*. 2007) or in the orchidectomized sand rat (BENMOULOUD *et al.* 2014). The hypertrophy of *zona reticularis* could be due to the need for androgens in order to cope with lack of gonadal steroids occurring after gonadectomy (KANDSI-BOUHADAD & HADJ-BEKKOUCHE 2010). Our results suggest an inhibitory effect of testicular androgens on glucocorticoid activity as reported by VIAU and MEANEY (1996) in laboratory rats.

Exogenous testosterone replacement reduced the pituitary adrenal axis activity. ACTH fell drastically, whereas the structure of the *zona fasciculata* was restored. Slight *zona reticularis* hyperplasia occurred since thick *zona* with small-sized cells was observed. The influence of testosterone on cortical cell remodeling may depend on the ability of androgen receptors localized in the adrenocortical zona to interact with the circulating testosterone hormone.

Several studies have shown that testosterone decreases the corticosterone response to stress *via* AR-mediated central mechanisms involving both CRH and AVP (VIAU & MEANEY 1996; LUND *et al*. 2004). In fact, the CRH contents and expression, as well as the number of immunoreactive neurons, increased in gonadectomized male (SEALE *et al*. 2004b). At the pituitary level, the overproduction of ACTH observed in the gonadectomized jird was probably driven by impaired negative feed-back of testosterone through the HPA axis.

Numerous data have shown the inhibitory effect of androgens on adrenocorticotropic hormone (AJDZANOVIC *et al*. 2015; HANDA *et al*. 1994a) and the level of expression of the POMC transcript (HANDA *et al*. 1994b; SHARMA & CHATURVEDI 2011). However, the mechanism by which testosterone acts remains unclear. Few studies have reported the expression of AR immunoreactivity in pituitary cells, especially in corticotroph cells. Indeed, some previous studies have shown that few if any ARs were present in corticotrophs (SHERIDAN & HERBERT 1980). O'HARA *et al*. (2015) also reported an increase in the percentage volume of the corticotroph pituitary cell type in AR knock-out mice. However, others studies have reported the colocalization of ARs with corticotroph cells, suggesting the direct effect of androgens on corticotroph function although the molecular mechanism remains unclear (MAEJIMA *et al*. 2009).

In our rodent model, further investigations are necessary to emphasize the localization of the androgen receptors at the corticotroph level. We also believe that the presence of androgen receptors in the three adrenocortical layers is largely involved in the structural remodeling of the gland rather than the regulation of steroidogenesis which may be regulated by pituitary ACTH. This fact does not exclude the role of testicular androgens on steroidogenesis by interfering with other factors which deserve to be studied.

Some papers have shown that testicular androgens can act either on adrenocortical enzymes, such as 21 and 11β-hydroxylases (PROVENCHER *et al*. 1992), 3βHSD (STALVEY 2002), or on growth factors. Recently JOPEK *et al*. (2017) showed that testosterone replacement stimulates expression of numerous genes in the rat, mainly those associated with lipids and cholesterol metabolism. Testosterone could also reduce adrenal sensitivity to ACTH (NOWAK *et al*. 1995) *via* ARs present in the adrenocortical cells (BENMOULOUD *et al*. 2014; ROSSI *et al*. 1998; TREJTER *et al*. 2015). In the desert rodent, *Psammomys obesus*, AMIRAT and BRUDIEUX (1993) reported a reduction in adrenal sensitivity to ACTH during spring when testosterone production was higher in this species (KHAMMAR & BRUDIEUX 1984). Such a phenomenon could also occur in the Libyan jird as suggested by immunostaining after testosterone treatment in all the adrenal *zonae*.

Recent studies have indicated that adrenal control is under the influence of various endocrine/paracrine factors, allowing the remodeling and zonation of the adrenal cortex. However, the molecular basis of this process remains unknown (PIHLAJOKI *et al*. 2015). Factors involved in this differentiation are expressed by stem/progenitor cells, but they can also be generated from other molecules. The stem/progenitor cells reside either in the adrenal capsule or in the sub-capsular region and can alter their shape, structure and hormone secretions in response to physiological needs and experimental manipulations.

In conclusion, this study provides further evidence that in the male Libyan jird inhabiting Sa-

hara desert areas, adrenocortical activity is negatively correlated with testicular activity during the annual reproductive cycle. The orchidectomy followed by testosterone replacement performed during the sexual season suggests a strong inhibitory action of the testicular androgens which act firstly at the pituitary level and also directly via their own receptors present in all adrenocortical *zonae*. These actions lead especially to the remodeling of the adrenal cortex activity, probably by triggering paracrine factors that drive the release of steroids required to maintain adequate adaptation in response to extreme physiological demands under arid environment.

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