Seasonal Changes in the Activity of the Adrenal Medulla of Viscacha (*Lagostomus maximus maximus*)

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ABSTRACT

Animals living in nontropical climates modify their physiology and behavior to adapt to seasonal environmental changes. Part of this adaptation involves the release of catecholamine from sympathetic nerve endings and the adrenal medulla, which play a major role in regulating energy balance. The aim of this work was to investigate whether adult male viscachas in their natural habitat exhibits structural changes in the adrenal medulla during the annual seasonal cycle. In August–September, chromaffin granules revealed ultrastructural changes suggestive of piecemeal degranulation. Quantitative morphometric analysis by transmission electron microscopy showed a significantly lower percentage of resting chromaffin granules and a higher percentage of altered granules and empty containers in August–September (late winter) compared to February–March (late summer), suggesting an increased secretory process of catecholamines in August–September. The mechanism of piecemeal degranulation might amplify this process, encouraging the adaptive response to winter environmental conditions. Tissue levels of epinephrine, norepinephrine, and dopamine (analyzed by high-performance liquid chromatography) changed throughout the year, reaching maximum values in February–March and minimum values in August–September. These results demonstrate morphological and biochemical seasonal variations of the adrenal medulla, suggesting that epinephrine might promote energy mobilization, which allow the *Lagostomus* to cope with adverse environmental conditions and thus to survive during winter season. Anat Rec, 296:1089–1095, 2013. © 2013 Wiley Periodicals, Inc.

Key words: adrenal medulla; epinephrine; chromaffin granules; *Lagostomus*

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In animals with seasonal reproduction, the allostatic response, that is, the changes occurring within the body for maintaining homeostasis and for coping with both predictable and unpredictable environmental challenges (McEwen, 1998; Landys et al., 2006; Stewart, 2006), involves the sympathoadrenal system and the hypothalamic-pituitary-adrenal axis. Activation of these systems induces the release of catecholamine from sympathetic nerve endings and the adrenal medulla, as well as glucocorticoids from the adrenal cortex (McEwen, 1998; McEwen and Wingfield, 2003). These hormones play a key role in the regulation of energy balance (McEwen, 2000).

Epinephrine stimulates lipolysis in adipose tissue and hepatic glycogenolysis, thus mobilizing stored energy (Chu et al., 2000, 2003; Demas and Bartness, 2001; Barth et al., 2007). In the lizard (*Podarcis sicula*), annual variations in epinephrine levels associated with the maintenance of skeletal muscle function and the establishment of social dominance during the breeding period have also been demonstrated (De Falco et al., 2004). Pyter et al. (2007) have reported that in male white-footed mice (*Peromyscus leucopus*), there are modifications in the hypothalamic-pituitary-adrenal axis that enable them to survive the short days of winter.

Our animal model, *Lagostomus maximus maximus* (viscacha), is a rodent mammal inhabiting semiarid zones in central Argentina and one of the largest rodents in the region (Weir, 1971). The male viscacha exhibits seasonal variation of gonadal activity in its annual reproductive cycle and in other metabolic functions that are under the influence of environmental signals such as photoperiod length, temperature, rainfall pattern, food composition, and social interactions. Thus, the reproductive activity of viscacha occurs during the long days of summer and early autumn, whereas during the short winter days, these animals experience significant testicular regression with the subsequent ceasing of the reproductive activity (Fuentes et al., 1993; Muñoz et al., 1997, 2001; Aguilera-Merlo et al., 2005; Filippa et al., 2005). In addition, an increase in the secretory ability of the pineal gland (Dominguez et al., 1987) and maximum levels of melatonin in the blood (Fuentes et al., 2003) have been determined in winter. Previous studies in adult male viscachas have shown morphological seasonal changes in the adrenal cortex, such as a decrease of nuclear volume and cellular activity in short photoperiod (Ribes et al., 1999). Environmental signals determine the beginning or end of the specific seasonal adaptations that allow the viscacha to maintain a positive energetic balance. In this way, the viscacha can adapt physiologically to seasonal climatic changes, undergoing the necessary endocrine adjustments needed for survival and for increasing its chances of reproductive success.

The aim of this study was to investigate whether the adrenal medulla of adult male viscachas in their natural habitat undergo structural changes throughout the year in relation to the periods of the annual reproductive cycle.

MATERIALS AND METHODS

Viscachas weighing 5–7 kg were captured in their habitat near San Luis, Argentina (33° 20' South latitude, 760 m altitude) during 2 consecutive years (2004–2005), using traps placed in their burrows. Solar irradiation values, expressed as heliophany, and monthly mean values of precipitations and temperature were provided by the Servicio Meteorológico Nacional San Luis (www.smn.gov.ar). In June–July, the lowest values of heliophany, precipitation, and temperature were observed (Table 1).

The seasonal study included a total of 30 animals, six animals per bimester: February–March (summer-early autumn, reproductive period), April–May (autumn, reproductive period), June–July (winter, gonadal regression period), August–September (late winter-early spring, gonadal recovery period), and October–November (spring, reproductive period). After being captured, the animals were immediately taken to the laboratory and anesthetized intraperitoneally with a ketamine (Ketamine Hydrochloride, Holliday Scott S.A.): xylazine (xylazine hydrochloride, Richmond Laboratories, Veterinary Division) solution (10:1, w/v, 0.3 mL/kg of body weight), sand sacrificed by intracardiac injection of euthanyle (0.25 mL/kg body weight, sodium pentobarbital, sodium diphenylhydantoin, Brouwer S.A.). The reproductive condition of viscachas was carefully assessed on the basis of light microscopy observations of testes. All the male viscachas captured in the June–July bimester were in the gonadal regression period. The left and right adrenal glands were quickly excised, weighed, and analyzed by histological, morphometric, and biochemical techniques. The left adrenal glands were used for histological and morphometric studies and immediately processed. The right adrenal glands were used for biochemical analysis and were stored at −70°C until being used. All animals were treated according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205) and following a study protocol approved by the Biodiversity Control Area of the Environment Ministry of San Luis, Argentina (Resolution No. 03 PRN-2011).

The adrenal glands were fixed in Bouin's fluid, dehydrated through an ethanol series, cleared in xylene, embedded in paraffin, and sectioned tranversally at 3–5 μm intervals. Every fifth section was stained with hematoxylin and eosin. The slides were mounted with Entellan (Merck, Darmstadt, Germany).

Electron Microscopic Study

The electron microscopic study was carried out in February–March and August–September. The adrenal glands

TABLE 1. Environmental conditions during the year

	February–March	April–May	June–July	August–September	October–November
Heliophany	9.38	7.09	6.11	7.21	9.09
Precipitation (mm)	90.00	27.00	11.00	15.00	58.50
Temperature (°C)	19.50	13.00	10.25	14.50	21.50

were immediately fixed in glutaraldehyde-paraformaldehyde solution in phosphate buffer, pH 7.4 (Karnovsky, 1965). The specimens were post fixed in 1% osmiun tetroxide for 2 hr at 4°C, dehydrated, and embedded in epoxy resin. Sections (1-μm thick) were stained with toluidine blue and examined by light microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate (Millonig, 1961), and observed under a Siemens Elmiskop I transmission electron microscope.

Quantitative Electron Microscopic Evaluation

The secretory activity of the adrenal medulla in February–March (late summer) and August–September (late winter) was evaluated by ultrastructural morphometric analysis of chromaffin granules. The study included determination of the percentage of granules with different ultrastructural features. The ultrastructural sections were randomly selected and photographed at ×3000 magnification. A total of 192 chromaffin cells were counted in each of the aforementioned bimesters (February–March and August–September). In each chromaffin cell were analyzed 30 granules. Thus, 5,760 granules were counted per bimester. Only cells with a large nucleus and abundant cytoplasm filled with chromaffin granules were chosen for quantitative analysis. Electron micrographs were scanned and converted into digital images. Higher magnification images of chromaffin cells were analyzed by Image Pro Plus 5.0 software. Three types of granules were counted: electron-dense chromaffin granules with the limiting membrane tightly adhered to the vesicle core (resting granules), granules with an eroded core surrounded by a light halo (altered granules), and granules with empty containers; the results were correlated to the net cytoplasmic area of each cell. The amount of each granule type was expressed as a percentage of the total number of granules.

Tissue Catecholamine Assay

Epinephrine, norepinephrine, and dopamine content in medullary tissue were measured by high-performance liquid chromatography (HPLC) with electrochemical detection, according to the method of Wagner et al., (1982) with some modifications.

Tissue samples were homogenized in ice-cold 0.2 M $HClO_4$ (15 μL/mg tissue) containing 0.3 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM $Na_2S_2O_5$, and 100 ng/mL of 3,4-dihydroxybenzylamine (as an internal standard) using an Ultra-Turrax homogenizer. After centrifugation for 20 min at 3000 rpm at 4°C, 300 μL of the supernatant were added to 300 μL of cold 1 M Tris HCl buffer (pH 8.6) and 5 mg of activated alumina. Catecholamines were adsorbed onto the alumina by shaking for 30 min and then centrifuged again. The supernatant was discarded and the alumina pellet was washed twice with cold ultrapure water. Catecholamines were eluted by agitation for 15 min with 100 μL of 0.2 M $HClO_4$. The eluent was stored at −70°C until analysis.

Aliquots (10 μL) of each sample were injected into the HPLC system, consisting of an ISCO 2350 pump, a Valco sample injector (model C6W), a Spherisorb ODS 2 reversed-phase column (5-μm particle size, 250 × 4.6 mm i.d.), and an L-ECD-6A electrochemical detector (Shimadzu model) with a glassy-carbon electrode. The mobile phase consisted of 0.02 M phosphate buffer (pH 3.0), containing 0.3 mM EDTA, 2% acetonitrile, and 0.12 mM sodium octylsulfonate. The mobile phase was filtered through a 0.45-μm Millipore filter and degassed under vacuum before use. The flow rate was set to 1.0 mL/min. The working electrode was operated at a potential of +0.6 V versus an Ag/AgCl reference electrode. Epinephrine (100 μg/mL), norepinephrine (100 μg/mL), and dopamine (100 μg/mL) stock solutions were prepared in 0.05 M $HClO_4$. Stock solutions were further diluted with 0.05 M HClO4 to obtain 100 ng/mL working solutions. The chromatograms were recorded and analyzed using Peak Simple II software. Results were expressed as nanograms of catecholamine per gram of wet tissue weight.

Statistical Analysis

Values were expressed as mean ± SEM Differences between groups were evaluated by analysis of variance (ANOVA). Post hoc comparisons between means were conducted using the Tukey–Kramer test when the overall ANOVA was significant. Morphometric data of electron microscopy were analyzed by unpaired Student's *t* test. A probability of less than 0.05 was assumed to be significant.

RESULTS

The bimesters studied in this work corresponded to the *Lagostomus maximus maximus* reproductive cycle periods. The body weight of viscachas was at its minimum value during the June–July bimester, although no significant differences were found ($P > 0.05$) compared with the other bimesters studied. Adrenal weight reached maximum values in February–March and minimum values in August–September (P 0.05, Table 2).

Electron Microscopic Observations

Because the analyzed biochemical parameters showed significant differences only between February–March and August–September, the ultrastructural features of the adrenal medulla were examined in these bimesters.

In February–March (late summer), chromaffin cells contained numerous secretory granules which occupied almost all the cytoplasm, and the granules displayed heterogeneous shapes and variable electron density. In addition, these cells exhibited a single nucleus of round to oval shape in a central or eccentric position, while the rough endoplasmic reticulum and the Golgi apparatus were localized in a paranuclear region with scarce or absent granules. A narrow extracellular space separated chromaffin cells from each other (Fig. 1).

In August–September (late winter), the general ultrastructural characteristics of the adrenal medulla were similar to those observed in late summer. However, chromaffin granules exhibited notable differences compared with February–March. Higher digital magnification revealed the presence of enlarged granule chambers and empty dilated containers. The content of some vesicles was reduced, appearing irregularly eroded with peripheral or internal areas of diminished electron density. Other vesicles were composed of a finely granular material. Small electron-dense vesicles were either attached

TABLE 2. Morphometric and biochemical seasonal changes in adrenal gland of *Lagostomus maximus maximus*

	February–March (N = 6)	April–May (N = 6)	June–July (N = 6)	August–September (N = 6)	October–November (N = 6)
Body weight (kg)	5.36 ± 0.28	4.80 ± 0.20	4.65 ± 0.15	4.67 ± 0.27	5.45 ± 0.25
Adrenal gland weight (g)	1.84 ± 0.07[a]	1.71 ± 0.05	1.53 ± 0.03	1.38 ± 0.06[b]	1.57 ± 0.09
Epinephrine (ng/g tissue)	747.59 ± 42.90[c]	673.83 ± 30.20	629.52 ± 24.93	515.28± 59.97[d]	605.93 ± 43.70
Norepinephrine (ng/g tissue)	126.72 ± 12.95[c]	110.22 ± 10.45	110.09 ± 13.58	75.42 ± 10.19[d]	102.97 ± 13.36
Dopamine (ng/g tissue)	22.38 ± 3.17[a]	21.78 ± 3.14	20.60 ± 3.02	12.58 ± 1.56[b]	13.89 ± 1.59

The values are expressed as mean ± SEM. Significant differences between February–March and August–September.
[a]versus
[b]*P* 0.05
[c]versu
[d]*P* 0.01 (ANOVA, Tukey–Kramer post hoc test).

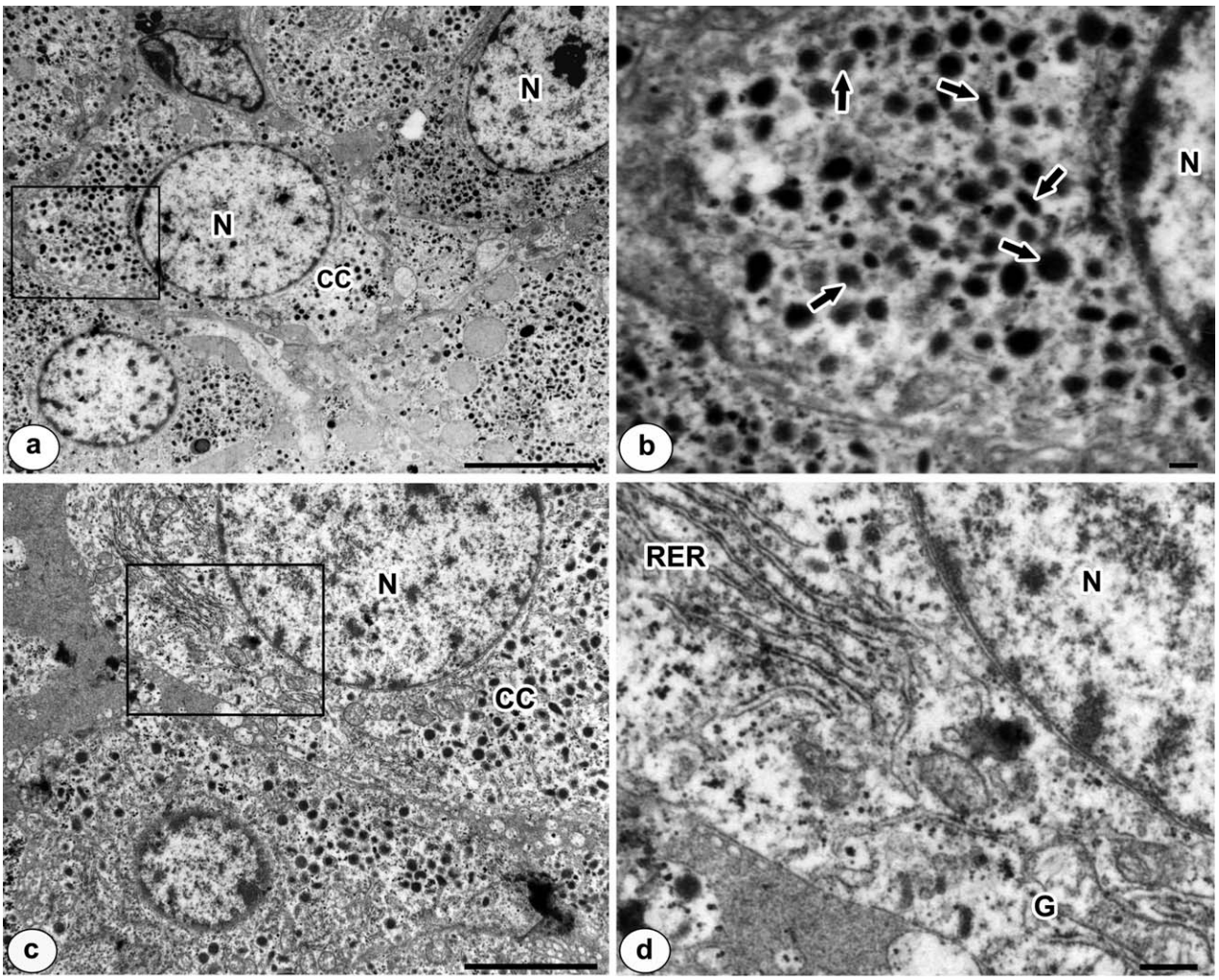

Fig. 1. Ultrastructural features of the adrenal medulla of *Lagostomus maximus maximus* in February–March (late summer). (**a**) Lower magnification image of chromaffin cell (CC); Scale bar: 10 μm. (**b**) Higher magnification image of the rectangle area of (a). There are granules with heterogeneous shapes and variable electron density (arrows). Scale bar: 100 nm. (**c**) Another lower magnification image. Scale bar: 5 μm. (**d**) Higher magnification image of the rectangle area of (c). It shows the rough endoplasmic reticulum (RER) and Golgi apparatus (G) in a chromaffin cell. Scale bar: 200 nm. N, chromaffin cell nuclei.

to the granule membrane or free in the cytoplasm. These vesicles were distributed near the chromaffin granules with scarce or absent core material. Electron-dense vesicles apparently bud from the perigranule membrane. Altered granules were intermixed with normal granules (Fig. 2).

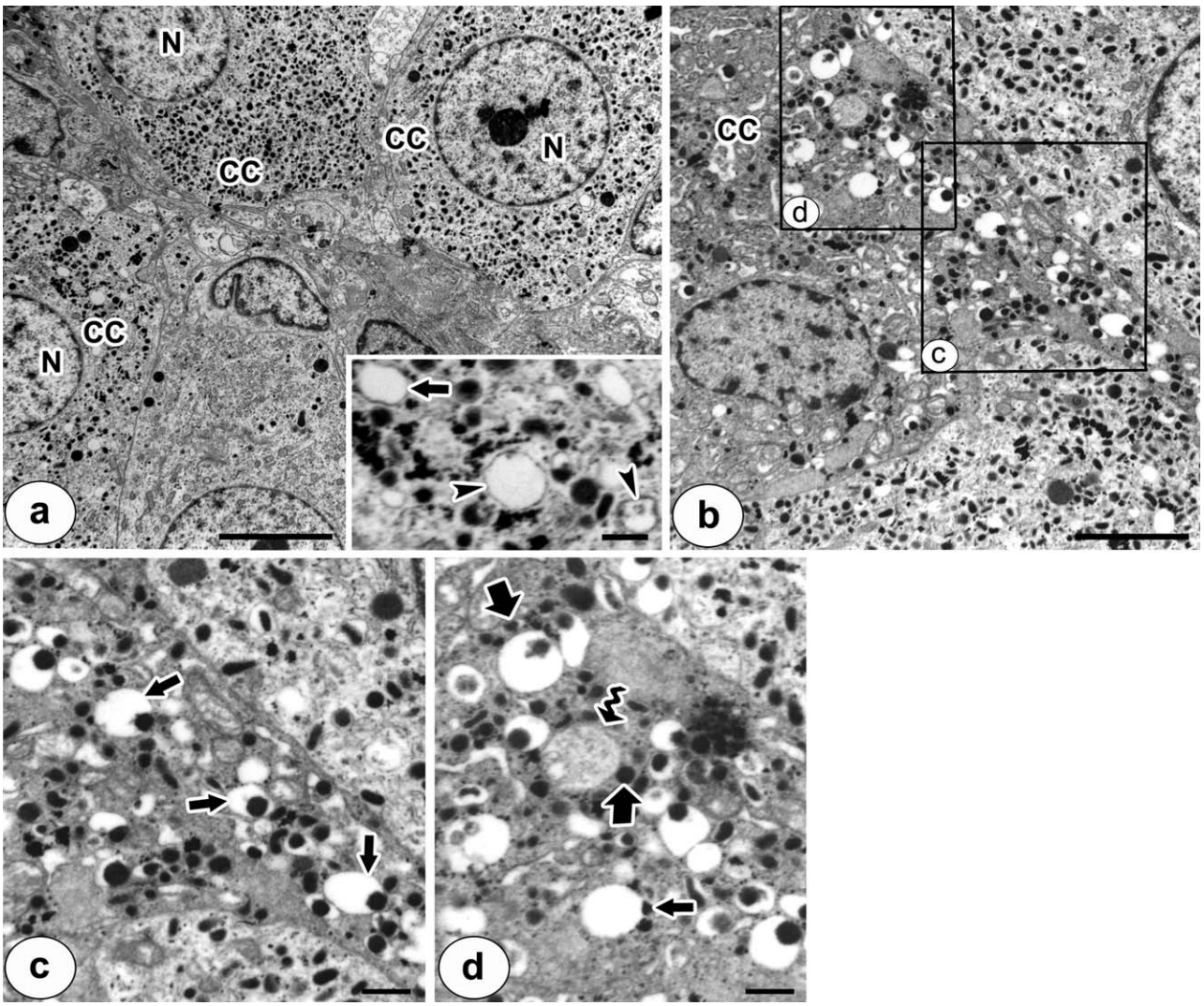

Fig. 2. Ultrastructural features of the adrenal medulla of *Lagostomus maximus maximus* in August–September (late winter). (**a**) Lower magnification image of chromaffin cells (CC). Scale bar: 10 μm. **Inset:** Higher magnification of a cytoplasmic part, showing empty dilated containers (arrow). Some granules show reduced content with peripheral or internal areas of diminished electron density (arrowheads). Scale bar: 150 nm. (**b**) Lower magnification image of chromaffin cell (CC). Scale bar: 7.5 μm. (**c**) Higher magnification image of the rectangle area of figure (b). It shows secretory vesicles with enlarged granule chambers and reduced content (arrows). Scale bar: 200 nm. (**d**) Higher magnification image of the rectangle area of figure (**b**); there are granules with a finely granular material (zig-zag arrow); some electron-dense vesicles are attached to the granule membrane (thin arrow) or are free in the cytoplasm, near the vesicles with scarce or absent core material (thick arrows). Scale bar: 200 nm. N, chromaffin cell nuclei.

The percentage of electron-dense chromaffin granules with the limiting membrane tightly adhered to the vesicle core (resting granules) was significantly lower in August–September than in February–March. On the other hand, chromaffin cells exhibited a significantly higher percentage of granules with an eroded core surrounded by a light halo (altered granules) and of empty containers in comparison to February–March (Fig. 3).

Annual Variations in Tissue Catecholamines

Levels of epinephrine, norepinephrine, and dopamine in tissue showed maximum values in February–March, and slowly decreased from April to August–September, when they reached minimum values. Tissue catecholamine levels increased again in October–November. Differences were significant only between February–March and August–September (Table 2).

DISCUSSION

The present study demonstrates the morphological and biochemical seasonal changes in the chromaffin cells of the viscacha adrenal medulla. This rodent lives in a semiarid subtropical climate and the hypothalamic-pituitary-adrenal axis is part of a general adaptation mechanism of several animals under adverse survival conditions. During winter, viscachas live under certain environmental conditions such as a short photoperiod, low temperature, hydric restriction, and food variations

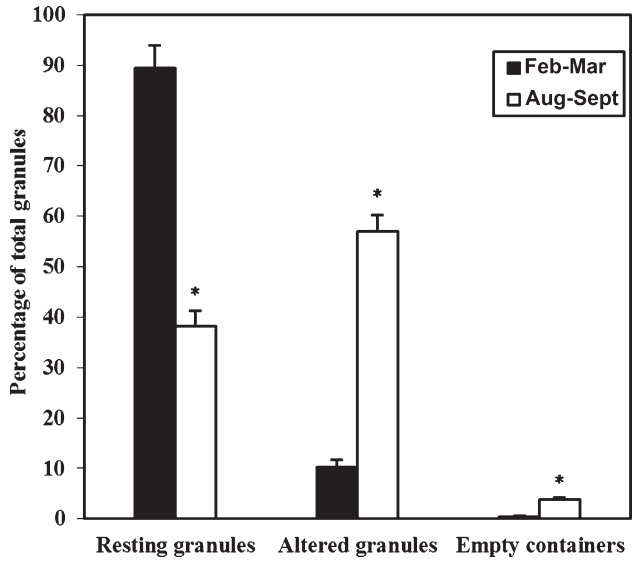

Fig. 3. Differences in chromaffin granule features between February–March and August–September. Values are expressed as mean ± SEM. Asterisks indicate significant differences between August–September and February–March (*t* test *P* 0.01).

(Branch et al., 1994; Bontti et al., 1999). These stressors are responsible for the increase in the synthesis of glucocorticoids represented by the high levels of serum corticosterone found in winter in adult male viscachas, when these rodents are in their gonadal regression period. Besides, ultrastructural studies of the fasciculata zone of the adrenal cortex demonstrated that the organelles involved in the synthesis of the steroid hormones were well developed during February–March (summer, reproductive period). On the contrary, cellular exhaustion was observed during August after the synthesis and release of glucocorticoids (Ribes et al., 1999).

In August–September, the chromaffin cells of the viscacha adrenal medulla displayed granules with content loss, suggesting a reduction of the catecholamine stored within secretory vesicles. These cells exhibited ultrastructural features suggestive of piecemeal degranulation, according to studies by Crivellato et al., (2003; 2006b). Morphometric analysis showed a significant reduction in the percentage of resting granules in August–September compared with February–March. On the other hand, in August–September chromaffin cells exhibited an increase in the percentage of both enlarged granules with an eroded core and empty containers, which are probably generated by the mobilization of granule contents. Therefore, the piecemeal degranulation process, which constitutes an alternative pathway for catecholamine secretion, was significantly higher during late winter and could promote the adaptive response to winter environmental conditions.

Piecemeal degranulation has been reported in the chromaffin cells of rat, mouse, and human adrenal medulla. This is a slow process implicated in the selective release of certain granule components over a period of hours or days. This alternative secretory route amplifies the endocrine response to stress in adrenomedullary chromaffin cells. The small electron-dense granules found in the cytoplasm might constitute vesicles that bud from the perigranule membrane and move toward the plasma membrane to discharge their contents. Detachment of these vesicles filled with electron-dense material might lead to the formation of empty or partially empty containers (Crivellato et al., 2004, 2006a).

The adrenal medulla secretes epinephrine and norepinephrine, although circulating norepinephrine derives mainly from sympathetic nerve endings (Nankova and Sabban, 1999; Carrasco and Van de Kar, 2003; Fulop et al., 2005). For this reason, tissue catecholamine levels were determined to fix their adrenomedullary origin. A significant reduction was observed in tissue catecholamine values of viscacha samples during August–September (late winter) compared with February–March (late summer). Thus, these biochemical findings agree with the ultrastructural observations and with the trend toward less body weight in winter compared to summer. These facts suggest that the secretory process of catecholamines might increase, allowing the viscacha to adapt physiologically to seasonal climatic changes by undergoing the necessary metabolic adjustments needed for survival and for increasing its chances of reproductive success.

The weight of adrenal glands reached a nadir in late winter, coinciding with the lowest annual values of tissue catecholamines found during that period. These results suggest that the higher secretory activity of the gland in late winter leads to increased catecholamine secretion, thus decreasing hormonal storage. Moreover, similar results were obtained for the adrenal cortex, where the nuclear volume of cortical cells decreased significantly in late winter (Ribes et al. 1999). Corticosterone serum levels showed a significant increase during winter, indicating hormonal tissue depletion.

Studies in different species have shown seasonal variations of catecholamine levels in the tissues related to temperature acclimation and the reproductive cycle (Harri, 1972; Osada and Nomura, 1989). Bartness et al., (2002) reported for Siberian hamsters an increase in the splanchnic drive to the adrenal medulla and release of catecholamine during short photoperiod. In addition, it has been demonstrated that, during the winter, high energy requirements and increased catecholamine secretion are necessary to guarantee an adequate energy supply for the tissues (Nelson and Draze, 2000).

Finally, seasonal variations in the activity of the adrenal medulla could be related to the adrenal cortex activity previously demonstrated for the viscacha. It has been reported that in other species epinephrine is able to stimulate corticosteroid production through a positive feedback, and therefore the adrenocortical function interacts with the gonadal axis in both a complementary and a reciprocal manner. This interaction appears to support reproductive and energy procurement strategies (De Falco et al., 2004). The reproduction of this animal is similarly regulated by the adaptation to the environment. Thus, a significant decrease in the activity of neuroendocrine reproductive control and in the testicular and epididymal function was found (Fuentes et al., 1993; Muñoz et al., 1997, 2001; Aguilera-Merlo et al., 2005).

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