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Annual variations of adrenal gland hormones in the lizard *Podarcis sicula*

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Abstract The adrenal gland regulates metabolism and maintains normal electrolyte balance. Adrenal hormones are equivalent in all vertebrates; the chromaffin tissue produces adrenaline and noradrenaline and the steroidogenic tissue produces most of the steroid hormones present in mammals. *Podarcis sicula* belongs to the Squamata family of lizards and it is the most abundant lizard species in southern Italy. This species shows a reproductive annual cycle and the presence of seasonal variations in the activity of the hypothalamus-hypophyseal-thyroid axis. To investigate the existence of an annual cycle of lizard adrenal gland, we have measured plasma concentrations of corticosterone, ACTH, noradrenaline and adrenaline. We have shown that corticosterone rapidly increased from January to March to reach a peak value that persisted until July, then, it slowly decreased until December. ACTH levels increased from January to May and slowly decreased from July to December. Noradrenaline levels were higher in March and then decreased until December. On the contrary, adrenaline levels increased from March to July and slowly decreased until December. Our results demonstrate the existence of an annual cycle of the lizard adrenal gland activity. This is very interesting because its activity is important to rightly regulate the reproductive status of the *Podarcis sicula* lizard.

Keywords Adrenal gland · Lizard · Plasma hormones

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Introduction

The adrenal gland has the important function of maintaining the homeostasis of the organism and to make appropriate changes in its physiology in response to acute stress, injury, etc. In some vertebrate classes, as in Reptilia, the gland lacks clear anatomical relationship with the kidney, being in close contact with the gonads and the gonoducts, and being only in proximity to the kidneys (Varano and Laforgia 1991). Reptilian adrenal glands do not have a uniform morphological organization: in some cases, the chromaffin tissue is almost completely separated from the steroidogenic cords and forms a compact dorsal mass; in other cases, the catecholamine cells form a continuous envelope around the steroidogenic parenchyma, with the envelope sometimes sending digitations toward the center of the gland. Finally, the dorsal chromaffin ribbon can be greatly reduced with most of the chromaffin cells arranged to form islets scattered in the steroidogenic parenchyma (Varano and Laforgia 1991).

Although the anatomy is very different among classes of vertebrates, from a physiological point of view, adrenal hormones produced are equivalent in vertebrates, in fact the chromaffin tissue produces both adrenaline and noradrenaline and the steroidogenic tissue produces most of the steroid hormones which are present in mammals.

Podarcis sicula belongs to the Squamata family of lizards. This lizard is the most abundant species in southern Italy. Like most saurians inhabiting temperate zones, this species shows a reproductive annual cycle well documented on the basis of both morphological (Angelini et al. 1976, 1986; Botte and Angelini 1980; Angelini and Ghiara 1984; Angelini and Botte 1992; Cardone et al. 2000, 2002) and endocrinological (Botte and Angelini 1980; Ciarcia et al. 1986; Andò et al. 1990, 1992; Gobbetti et al. 1993a, b) parameters. In early spring, the lizards emerge from winter shelters;

gonads and secondary sexual characters (SSC) begin to develop and are functional until the end of June to the beginning of July. From March to April male lizards are engaged in fights (aggressive phase), which are linked to reproductive territory assessment. At the end of April to the beginning of May, courtship and mating begin and last for several weeks (mating phase). Female lizards have a single ovulatory wave in spring–summer and it is preceded by typical morpho-physiological modifications of the ovary that include an increase in the number and size of follicles, and the onset of vitellogenesis (Filosa 1973; Borrelli et al. 2000). In July, when the temperature should be still favorable to reproduction, a refractory period occurs, inducing a block of spermatogenesis and the regression of SSC (refractory phase). In autumn, spermatogenesis is resumed and some sperms are produced but there is no spermiation and SSC do not develop. From November to March, ambient temperature declines and the lizards undergo semi-hibernation (Manzo et al. 1994). In females, at the end of the ovulatory period, the ovary enters a fall–winter stasis that lasts until the next spring (Filosa 1973; Borrelli et al. 2000). The *Podarcis sicula* adrenal gland is under hypothalamus–hypophyseal axis control (Laforgia and Varano 1978; Laforgia and Muoio 1997). It has been demonstrated that ACTH administration enhances adrenaline synthesis (Basserji et al. 1986; Kent and Parker 1993; Laforgia and Muoio 1997). Moreover, some authors have demonstrated that in hypophysectomized animals there is an increased degradation of phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme responsible for the conversion of noradrenaline to adrenaline through methylation (Basserji et al. 1986; Jiang et al. 1989; Bornstein et al. 1991; Kent and Parker 1993; Laforgia and Muoio 1997). It has been demonstrated that chromaffin tissue does not show evident morphological changes during the annual reproductive cycle. On the contrary, steroid-producing cells morphologically appear less stimulated in winter (January–February) than in the spring–summer–autumn seasons. In addition, Leydig cell activity in androgen synthesis is strictly related to the plasma corticoid levels secreted by the adrenal gland, particularly under stress (Manzo et al. 1994). Therefore, the gonad-epididymis-adrenal complex can be considered as an integrated system in the regulation of reproductive activity in reptiles (Manzo et al. 2000). It has been demonstrated that embryologically there is a significant relationship between ontogenic steroidogenesis of the adrenal gland and sexual differentiation of the gonad (Doddamani 2000).

In the light of the above data, it is evident that adrenal glands interact with different endocrine systems such as gonads and the thyroid gland to control basal homeostasis. The aim of the present study was to investigate the annual variations of plasma concentration of steroid and catecholamine hormones in the lizard *Podarcis sicula*.

Materials and methods

Animals and experimental design

Adult specimens of *Podarcis sicula* (12–15 g body weight) of both sexes were captured in the neighborhood of Naples. After capture, the animals were housed in large soil-filled terraria containing heather, and exposed to natural temperature and photoperiod. Water dishes were present in the terraria, and the animals were fed on live fly larvae daily. In order to study annual variation in the adrenal gland function, on the 15th day of each month, at 8:00 a.m., a group of 15 animals (8 male and 7 female) was killed by decapitation 2 days after the capture. This experimental design was conducted for 2 consecutive years. Even if the blood sampling is usually carried out from caudal vein or retroorbital sinus, decapitation was done to let the collection of blood samples immediately after capture to minimize adrenal secretion due to handling. All animals have been captured with the authorization of 06/01/2000 n. SCN/2D/2000/9213 of the Italian Ministry of Environment. All animal experimentations were conducted in accord with accepted standards of human animal care.

Hormone assay

Blood samples were collected by intracardiac puncture and put into heparinized capillaries. They were then centrifuged at 1,500 rpm for 10 min to obtain serum.

Corticosterone was measured in 100 µl of plasma using a sensitive and highly specific radioimmunoassay kit (ICN BIOMEDICAL, Costa Mesa, Calif., USA). Before assay, plasma samples were diluted one-third with the kit diluent and heated at 80°C for 10 min in order to inactivate corticosterone-binding proteins. Corticosterone titers in picograms per milliliter were calculated using the standard curve generated in the assay. Standard curves were prepared in buffer with known amounts of radio inert corticosterone (0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 pg ml^{-1}) purchased from Amersham Corp. (Arlington Heights, Ill., USA). Sensitivity was 0.176 pg ml^{-1}. Cross-reactivities of the corticosterone antiserum with other steroids were 6.1% for deoxycorticosterone; $<1\%$ for progesterone and cortisol; $<0.1\%$ for aldosterone, 20-α dehydroprogesterone, testosterone and 11-deoxycortisol; and $<0.01\%$ for all the other steroids examined. Inter- and intra-assay coefficients of variation were 5.8 and 3.4%, respectively. Cross-reactivities of the ACTH antiserum were 0.03% for α-MSH, 0.01% for β-MSH and 0.02% for β-endorphin.

ACTH concentrations were measured in 100 µl of plasma by a two-site immunoradiometric assay (IRMA) using mouse monoclonal antibodies (DIAGNOSTIC Products) as previously described in De Falco et al. (2003). Boric acid was added to serum to adequately preserve ACTH. Sensitivity was 0.1 pg ml^{-1}, and the inter- and intra-assay coefficients of variation were 10 and 6%, respectively.

Catecholamines (200 μl) were extracted from plasma by the alumina adsorption method, and plasma adrenaline and noradrenaline levels were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (Woodward 1982). The HPLC system consisted of a Varian Star 9012 solvent delivery system (Varian Chromatography System, Walnut Creek, Calif., USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, N.J., USA). The alumina-extracted samples (200 μl) were passed through an Ultratechsphere ODS-C18 5 μm column (HPLC Technology), using a catecholamine and metanephrine mobile phase (Chromsystem Munich, Germany). The separated amines were integrated by the Star Chromatography software program (version 4.0; Varian). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylalamine hydrobromide (DHBA) as an internal standard in all determinations, with a detection limit of 0.1 nmol l^{-1}. Plasma catecholamine concentrations were expressed as pg ml^{-1}.

Statistical analysis for plasmatic hormone values

All data were expressed as means ± standard error of mean (SEM). Statistical analysis was performed by means of one-way analysis of variance (ANOVA) for repeated measures, with least squared means a post-hoc test, or unpaired Student's *t*-test. A two-tailed probability of less than 5% (i.e., $P < 0.05$) was considered statistically significant.

Results

In order to investigate the annual cycle of lizard adrenal gland, we have analyzed plasma concentrations of adrenal hormones: corticosterone and catecholamines, both noradrenaline and adrenaline. In addition, we have also measured plasma ACTH levels to verify the activity of hypothalamus-hypophyseal-adrenal gland axis during the year. We have performed these experiments for 2 consecutive years but no significant differences were shown between years. Values shown represent a mean of 2 consecutive years. In addition, no sex-related differences were observed in each experimental group for all hormones measured.

Plasma corticosterone levels rapidly increased from January (3.57 ± 0.2 pg ml^{-1}) to March (6.31 ± 0.3 pg ml^{-1}) when they reached the peak value that persisted until July. Then, corticosterone levels slowly decreased to reach the minimum value in November (5.32 ± 0.3 pg ml^{-1}) (Fig. 1). We have also measured plasma concentrations of ACTH and we have observed that this hormone had its minimum value in January (2.82 ± 0.1 pg ml^{-1}), slowly increased in March (4.40 ± 0.2 pg ml^{-1}), reached the peak value in May (6.27 ± 0.3 pg ml^{-1}) and slowly decreased from July (4.22 ± 0.2 pg ml^{-1}) to November (3.23 ± 0.2 pg ml^{-1}) (Fig. 1a).

With regard to catecholamines, plasma noradrenaline concentrations very rapidly increased from January (229 ± 11 pg ml^{-1}) to March (920 ± 40 pg ml^{-1}), slowly decreased in May (578 ± 28 pg ml^{-1}) and in July (613 ± 31 pg ml^{-1}) to reach a very low value in November (340 ± 16 pg ml^{-1}). A different annual course was observed for adrenaline. In fact, it had the minimum value in January (119 ± 6 pg ml^{-1}), less rapidly increased in March (500 ± 20 pg ml^{-1}) and finally reached maximal values in May (985 ± 48 pg ml^{-1}) and in July (998 ± 50 pg ml^{-1}). Adrenaline concentration then strongly decreased until November (205 ± 10 pg ml^{-1}) (Fig. 1b).

One-way ANOVA for repeated measures of the plasma corticosterone, ACTH and catecholamine concentrations in all months showed a significant difference between groups ($P < 0.001$); all other specific comparisons were derived from post-hoc tests ($P < 0.05$).

Discussion

The adrenal gland is mainly controlled by the hypothalamus–hypophyseal–adrenal axis and it functions in regulating metabolism and maintaining normal electrolyte balance. It secretes steroid and catecholamine (noradrenaline and adrenaline) hormones. The steroidogenic and the chromaffin tissues of reptiles are closely associated, but there is a considerable variation in the degree of admixing (Varano and Laforgia 1991). It has been demonstrated that differentiation of chromaffinoblasts into chromaffin cells is probably stimulated by the influence of hormones, which are produced by the cells of the interrenal tissue (Doddamani 2000; Rupik 2002). In Squamata most of the chromaffin tissue forms a noticeable dorsal mass which sends expansions to penetrate between the interrenal cords. Within the parenchyma there are numerous islets of chromaffin tissue. Chromaffin cells are so named because the catecholamines contained in their cytoplasmic granules have an affinity for chromium salts and darken on oxidation by potassium dichromate. It has been clearly demonstrated that in the mammalian adrenal gland, the relative quantities of adrenaline and noradrenaline depend strictly on the development and the activity of the adrenal cortex, as corticosteroids activate the PNMT enzyme (Coupland and McDougall 1966; Kirshneer and Goodall 1957; Pohorecky and Rust 1968). This influence on PNMT activity by corticosteroid hormones has been recently demonstrated also in *Podarcis sicula*. In turn, adrenaline is able to stimulate corticosteroid production through a positive feedback (Laforgia and Muoio 1997; De Falco et al. 2003). Therefore, the steroidogenic tissue is also under the control of hypothalamus–hypophyseal

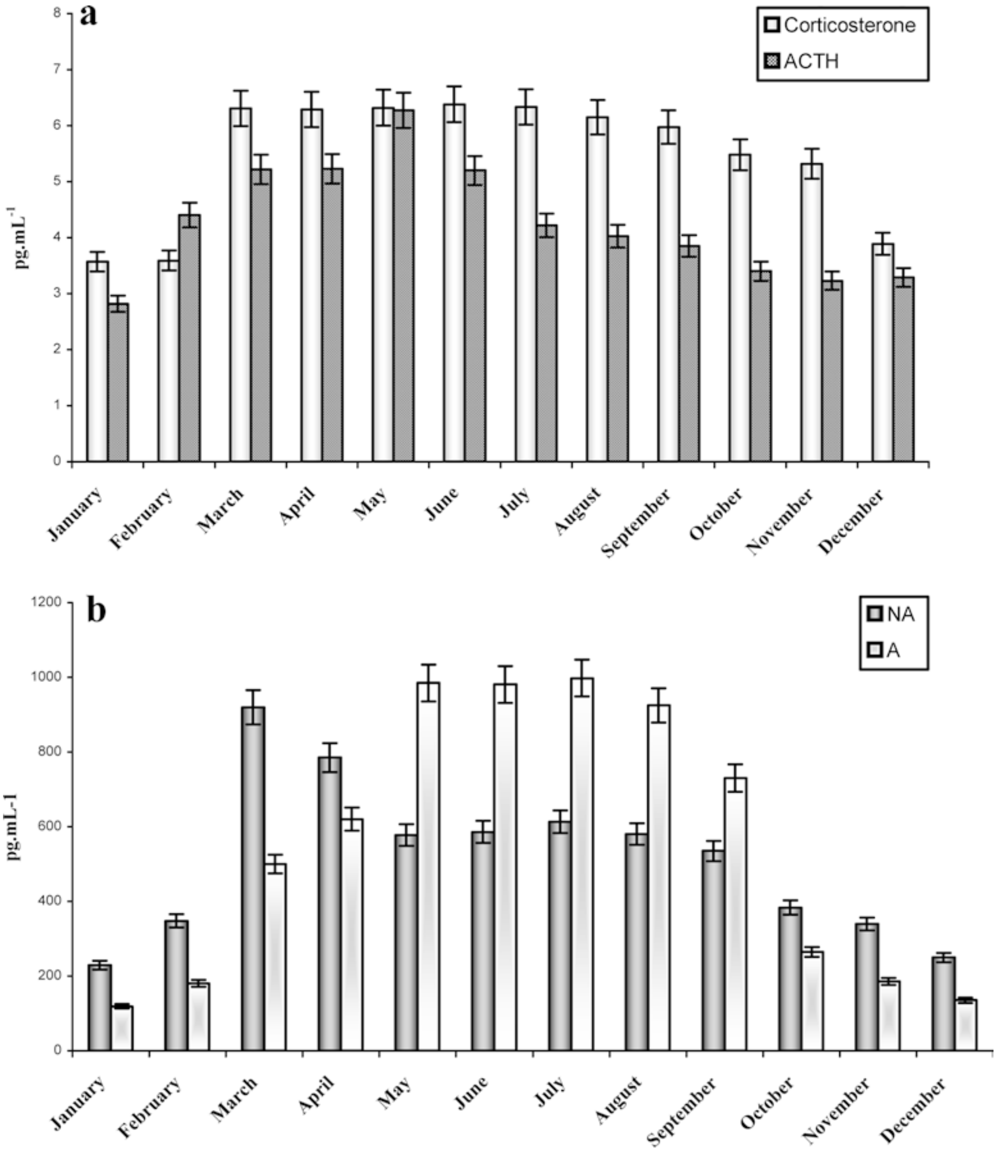

Fig. 1a, b Annual variations of *Podarcis sicula* adrenal hormones. **a** Annual time-course of plasma concentrations of corticosterone and ACTH levels. Values are mean ± SD obtained for 2 consecutive years (for each group $n = 15$). For all values, $P < 0.05$. **b** Annual time-course of plasma concentrations of catecholamines: noradrenaline and adrenaline. Values are mean ± SD obtained for 2 consecutive years (for each group, $n = 15$). For all values $P < 0.05$

axis through ACTH action (Laforgia and Varano 1978; Laforgia and Muoio 1997). Several evidences indicate that lizard adrenocortical function interacts with the gonadal axis in both a complimentary and reciprocal manner. This interaction appears to support reproductive and energy procurement strategies (Grassman and Crews 1989; Moore et al. 1991; Grassman and Hess 1992a, b; Manzo et al. 1994; Wilson and Wingfield 1994), territorial strategies (Knapp and Moore 1997), and social status (Greenberg et al. 1984). Indeed, the adrenal gland of some species may serve as an accessory source of progesterone and testosterone (Dauphin-Villemant and Xavier 1985; Dauphin-Villemant et al. 1990; Grassman and Hess 1992b; Manzo et al. 1994).

In order to investigate the existence of an annual cycle of *Podarcis sicula* adrenal gland, we have measured plasma concentrations of several hormones such as corticosterone, ACTH, noradrenaline, and adrenaline for 2 consecutive years. We have observed that both corticosterone and ACTH rapidly increased from January to March, when the lizards leave their winter shelters, and reached their maximal values in May. While ACTH concentrations decreased slowly from July to November, corticosterone levels remained high both in May and in July to decrease very slowly in November. We also didn't observe any difference between males and females as demonstrated by Carsia and John-Alder (2003). Corticosterone levels were probably increased due to adrenaline release by chromaffin cells. In fact, when we observed the variations of catecholamine concentrations, we have shown a very fast increase of noradrenaline from January to March and its slow decrease from May to November. On the contrary, adrenaline increased progressively from January to July, to finally decrease in November. In particular, decrease of noradrenaline concentration during March and July can be explained with the activation of PNMT, the enzyme that converts noradrenaline into adrenaline. Moreover, it has been demonstrated that the increase of the expression level of PNMT enzyme is induced by corticosterone (Laforgia and Muoio 1997).

In the last years, in reptiles, the presence of seasonal variations has been shown in the activity of the hypothalamus–hypophyseal–thyroid axis related to such factors as hibernation or reproduction, and influenced by climatic variations and temperature (Sciarrillo et al. 2000). In particular, temperature plays a very relevant role in the reproduction of *P. sicula* (Borrelli et al. 2000). However, this does not rule out the possibility that in this lizard, as in other ectotherms, the annual reproductive cycle is regulated by the interplay between environmental temperature and photoperiod (Vivien-Roels 1985; Borrelli et al. 2000). It has been demonstrated that higher seasonal levels of plasma corticosterone coincided with periods of peak energy demand: reproductive and territorial behavior in males (John-Alder et al. 1997), vitellogenesis and gestation in females (Carsia and John-Alder 2003). In other species, plasma corticosterone is also correlated with body condition (John-Alder et al. 2002); in both sexes, body condition, measured as mass per unit length, is relatively high at emergence from hibernation and remains so throughout the breeding season (John-Alder et al. 2002). Thereafter, body condition in both males and females declines from June through August, indicating a decline in stored energy relative to body length, and recovers to its highest annual level just prior to hibernation (Carsia and John-Alder 2003). The regulation of testicular activity depends on several endogenous factors, and many authors have demonstrated a close relationship between testicular, adrenal and thyroid functions (Cardone et al. 2000, 2002; Manzo et al. 2000; Sciarrillo et al. 2000). In particular, the highest levels of corticosterone occur during the mating phase (May). The involvement of corticosterone in reproduction was supported by its in vitro effect on testosterone release by the testis, since the latter hormone was dramatically reduced during aggressive and mating phase by corticosterone treatment (Manzo et al. 1994). Some studies have indicated that the levels of catecholamines are mainly important during the breeding period since plasma catecholamine were significantly elevated within 30 s in male combatants. In addition, it has been observed that at 30 s following the determination of the outcome, winning male lizards had plasma noradrenaline levels that were higher than those of the losers (Summers and Greenberg 1994). These aggressive interactions, in *Anolis carolinensis*, resulted in increased circulating levels of noradrenaline and adrenaline (Summers and Greenberg 1994). In addition, it has been recently shown that higher plasma adrenaline and noradrenaline levels were exhibited by males viewing an opponent with eyespots painted black in respect to males with eyespots hidden with green paint and isolated controls (Korzan et al. 2002). The darkening of the eyespot, from green to black, is a social signal that communicates disposition and determines social hierarchy. Hence, the catecholamines probably serve to determine the male that is more qualified for reproduction. Although most studies of the neuroendocrine bases of aggression focus on males, female aggression is common in many species and contexts. The aggressive behavior is common in competition for resources and is used to attain or maintain dominance rank (Andrews and Summers 1996) or to defend territories that may contain feeding or nesting sites (Gowaty and Wagner 1988; Jaeger et al. 1982; Woodley and Moore 1999). In other cases, aggression allows females access to mates or benefits provided by mates (Fivizzani and Oring 1986; Woodley et al. 2000). Woodley et al. (2000) have demonstrated that both males and females had increased plasma corticosterone after an aggressive interaction during the nonbreeding season (Woodley et al. 2000). It has been hypothesized that these physiological changes, in both sexes, can influence the expression of subsequent aggression (Haller et al. 1998; Schuett et al. 1996; Summers and Greenberg 1995; Wingfield et al. 1990; Woodley et al. 2000).

Moreover, it has also been demonstrated that plasma adrenaline, noradrenaline and corticosterone increased after 5 min of exhaustive exercise in the lizard *Dipsosaurus dorsalis*. Therefore, adrenaline had a stimulatory effect on lactate removal by skeletal muscle (Gleeson et al. 1993).

We have shown an annual cycle for corticosterone and ACTH levels that represent the annual activity of steroidogenic tissue and hypophysis, respectively. Moreover, we have also shown annual variations of catecholamine levels during the year. Intriguingly, we can hypothesize that corticosterone and adrenaline hormones influence each other. The maximal value of the corticosterone level in March can probably induce an increase of noradrenaline methylation with a consequent adrenaline production as previously demonstrated by Laforgia and Muoio (1997). Adrenaline, in turn, could stimulate corticosterone secretion.

Since the adrenal gland function is strictly (but not exclusively) related to gonads (Manzo et al. 2000), adrenal hormone variations during the year have to be considered important in a seasonal breeding lizard such as the *Podarcis sicula*. Therefore, the increase of adrenaline from March to July is very important to maintain a good function of skeletal muscles and to establish social dominance relationships that are of fundamental importance for reproduction. Further studies will help to clarify adrenal function in the contexts of organismal energetics, and physiological and behavioral aspects of reproductive biology.

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