General and Comparative Endocrinology 158 (2008) 202–210

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Gonadal modulation of *in vitro* steroidogenic properties of dispersed adrenocortical cells from *Sceloporus* lizards

Rocco V. Carsia [a], Patrick J. McIlroy [b], Robert M. Cox [c], Michele Barrett [d], Henry B. John-Alder [e,*]

[a] Department of Cell Biology, University of Medicine and Dentistry of New Jersey, School of Osteopathic Medicine, Stratford, NJ 08084, USA
[b] Department of Biology, Camden College of Arts and Sciences, Rutgers University, Camden, NJ 08102, USA
[c] Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA
[d] Cornell University, College of Veterinary Medicine, Ithaca, NY 14853, USA
[e] Department of Ecology, Evolution, and Natural Resources, Rutgers University, 14 College Farm Road, New Brunswick, NJ 08901, USA

ARTICLE INFO

Article history:
Received 23 February 2008
Revised 23 June 2008
Accepted 17 July 2008
Available online 3 August 2008

Keywords:
Lizard
Adrenal
Adrenocortical cell
Gonad
Corticosteroid
Aldosterone
Corticosterone
Progesterone

ABSTRACT

Effects of adrenal corticosteroids on reproductive and endocrine functions of the gonads are well known, but reciprocal effects of gonadal hormones on the hypothalamo-pituitary-adrenal (HPA) axis and on adrenocortical steroidogenesis in particular have received much less attention. We investigated effects of gonadectomy and testosterone (T) replacement on adrenocortical cell function in a year-long field study of male *Sceloporus undulatus* (Eastern Fence Lizard) and in a shorter term laboratory study with male *Sceloporus jarrovii* (Yarrow's Spiny Lizard). We also compared females to males in *Sceloporus virgatus* (Striped Plateau Lizard) and investigated effects of gonadectomy in short-term laboratory experiment on females of this species. As measured by *in vitro* production of progesterone (P_4), corticosterone (B), and aldosterone (ALDO), sensitivity of adrenocortical cells to corticotrophin (ACTH) was lower in control males than females of *S. virgatus*. In *S. jarrovii* males, cellular sensitivity to ACTH was reduced by orchiectomy but was not restored to levels of intact controls by T replacement. By contrast, in *S. undulatus*, cellular sensitivity to ACTH was not affected by orchiectomy alone but was reduced by T replacement in orchiectomized males. Maximal rates of steroid production were less consistently affected by experimental treatments, but were lower in males than in females of *S. virgatus* and were dramatically reduced by T replacement in orchiectomized *S. undulatus* males. Overall, our experiments clearly demonstrate two distinct sources of variation in functional capacities of dispersed adrenocortical cells isolated from *Sceloporus* lizards: (1) naturally occurring differences between males and females (Carsia and John-Alder, 2003), and (2) species-dependent changes in response to surgical gonadectomy with or without exogenous testosterone. Sex differences and functional lability in adrenocortical cells are probably widespread among vertebrates and may be an important component of variation in output of the HPA.

1. Introduction

Functional cross-talk is well-documented between the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-gonadal (HPG) axes, especially with respect to inhibition of the HPG axis by adrenal glucocorticoids (Rivier and Rivest, 1991; Sapolosky et al., 2000; Tilbrook et al., 2000). However, reciprocal effects of gonadal hormones on the HPA axis are less widely known (see Viau, 2002). Studies on mammals indicate that the gonads can affect the HPA axis at all levels (see Malendowicz, 1994; Dallman et al., 1992). Gonadal effects on the HPA axis seem to be mediated primarily by gonadal steroids (Viau, 2002), but non-steroidal gonadal factors may also have a role (Kitay et al., 1966).

In free-living vertebrates, variation in adrenal glucocorticoid output commonly occurs in association with reproductive maturation (see McQuillan et al., 2003) and annual breeding cycles (Romero, 2002), an observation that implicates gonadal regulation of HPA function. In birds, for example, most evidence in support of this conjecture is largely circumstantial, derived from correlations between sex, reproductive and seasonal parameters, and circulating corticosterone levels (see Carsia and Harvey, 2000). Sex differences at the cellular level are clearly indicated by modest sex differences in the steroidogenic response to ACTH by adrenocortical cells from domestic fowl (*Gallus gallus domesticus*) (Carsia et al., 1987a,b), Japanese quail (*Coturnix coturnix japonica*) (Carsia et al., 1988) and turkeys (*Meleagris gallopavo*) (Kocsis and Carsia, 1989). However, few studies have definitively shown a role of the gonads in the regulation of the avian adrenal steroidogenic function by using the standard endocrine model of gonadectomy with or

* Corresponding author. Fax: +1 732 932 8746.
E-mail address: henry@aesop.rutgers.edu (H.B. John-Alder).

0016-6480/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2008.07.018

without sex steroid replacement. Although earlier work showed that tonically administered gonadal steroids suppress adrenocortical function in chickens (Kar, 1947; Nagra et al., 1965), subsequent studies with orchiectomized chickens produced equivocal results, showing that effects of orchiectomy appear to be conditionally dependent upon strain, age, and duration of the orchiectomized state (Chester Jones, 1957). More recent work with dispersed adrenocortical cells derived from orchiectomized cockerels suggests that androgen maintenance suppresses the adrenocortical steroidogenic response to ACTH (Carsia et al., 1987a,b). In birds as in mammals, it can be inferred that the gonads may influence adrenocortical activity by regulating the release of both inhibitory and trophic substances. Additionally, since sex differences in steroidogenic responses to ACTH are apparent in adrenocortical cells prior to sexual maturity (Carsia et al., 1987a,b; Kocsis and Carsia, 1989), a gonad-independent effect (Arnold and Burgoyne, 2004) is also plausible.

Given that birds constitute a derived reptilian lineage, lizards, and other reptiles might plausibly exhibit similar gonadal influences on the HPA axis. However, ectothermy and other fundamental physiological traits of non-avian reptiles may impose unique constraints on the interaction between the HPA and HPG axes. Furthermore, since the adrenal may serve as an auxiliary source of progesterone and testosterone in some reptiles (Dauphin-Villemant and Xavier, 1985; Dauphin-Villemant et al., 1990; Grassman and Hess, 1992; Manzo et al., 1994), gonadal effects on the adrenal gland may differ between birds and other reptiles. However, reciprocal influences between the HPG and HPA in reptiles have yet to be definitively investigated by combining orchiectomy and sex steroid replacement with direct assessment of adrenocortical function.

The use of freshly dispersed adrenocortical cells has aided our understanding of the regulation of mammalian and non-mammalian adrenal steroidogenesis. Our recent characterization of adrenocortical cells derived from the Eastern Fence Lizard (*Sceloporus undulatus*) (Carsia and John-Alder, 2003) provided a starting point for examining the influence of the gonads on reptilian adrenal steroidogenesis at the cellular level. In the present study, we report the influence of gonadectomy on adrenal steroidogenesis using freshly dispersed adrenocortical cells derived from three species of *Sceloporus* lizards. The experiments reported here took advantage of opportunities to investigate functional properties of dispersed adrenocortical cells harvested from three species of *Sceloporus* lizards that had been used in separate studies on growth regulation (Carsia et al., 2004; Cox et al., 2005, 2006). We report unambiguous evidence that adrenal cellular steroidogenic responses depend on gonadal function in lizards. However, gonadal effects on adrenocortical cells are complex and dependent on species, sex, and experimental and environmental conditions.

2. Materials and methods

2.1. Field experiment

Juvenile male Eastern Fence Lizards (*S. undulatus*; ca. 10 months of age) were collected in the pinelands of New Jersey (approximately 40°N, 74°30′W) under permit from the New Jersey Department of Environmental Protection, Division of Fish and Wildlife (permit SC 22053) and were temporarily housed in the laboratory, where they were provided food (crickets, *Acheta domestica*) and water ad libitum and assigned to one of three treatment groups. Intact males receiving sham surgery and intraperitoneal placebo implants served as controls (CON). The two remaining groups were surgically orchiectomized (Cox and John-Alder, 2005) and received either placebo implants (ORCHX) or testosterone implants (ORCHX + T). Testosterone implants consisted of Silastic® tubing (Dow Corning, Midland, MI, USA; 1.47 mm id, 1.96 mm od) containing 300 μg of crystalline testosterone (Sigma T-1500, Sigma–Aldrich Inc., St. Louis, MO, USA) within a 1.5-mm lumen sealed by silicone adhesive (Cox et al., 2005). One day after completion of surgical treatments, lizards were returned to a field enclosure adjacent to the Rutgers Pinelands Research Station, where they remained until their recapture 441 days later (ca. 22 months of age). Blood samples were collected in the field upon recapture for measurement of plasma testosterone to verify the effectiveness of experimental treatments. Methods and results of the testosterone radioimmunoassay have been reported previously (Cox et al., 2005). Lizards were then transported to the laboratory at Rutgers University where they were held overnight, killed by decapitation, and immediately necropsied for adrenal glands.

2.2. Laboratory experiments

Juvenile male Yarrow's Spiny Lizards (*Sceloporus jarrovii*; 3–4 months of age) were collected near Buena Vista Peak in the Chiricahua Mountains, Coronado National Forest, AZ, USA (approximately 31°54–55′N, 109°16′W) under permit from the Arizona Game and Fish Department (permits SP 751920 and 553889) and were transported to the laboratory at Rutgers University, where they were provided food (crickets, *A. domestica*) and water ad libitum. Lizards were surgically orchiectomized and received intraperitoneal placebo implants (ORCHX) or implants containing testosterone (ORCHX + T), as above. Intact males receiving sham surgeries and placebo implants served as controls (CON) (Cox et al., 2006). After six weeks of treatment, lizards were killed by decapitation and their carcasses were immediately necropsied for adrenal glands. Blood samples for measurement of plasma testosterone were collected from the neck following decapitation to verify the effectiveness of treatments. Methods and results of the testosterone radioimmunoassay have been reported in Cox et al. (2006).

Striped Plateau Lizards (*Sceloporus virgatus*; ca. 8 months of age) were collected in the Chiricahua Mountains, Coronado National Forest, Arizona, USA (approximately 31°54–55′N, 109°16′W) under permit from the Arizona Game and Fish Department (permits SP 751920 and 553889) and were transported to the laboratory at Rutgers University, where they were provided food (crickets, *A. domestica*) and water ad libitum. Experimental females (OVX) were surgically ovariectomized as described for *S. jarrovii* in Cox (2006). Intact females received a sham surgery and served as controls (FEM). Intact males (MALE) of *S. virgatus* were also included for a comparison of adrenocortical function in females versus males. After six months in captivity following surgery, lizards were killed by decapitation and their carcasses were immediately necropsied for adrenal glands. Blood was not collected for hormone assays from this species. However, levels of plasma T are substantially higher in males than in females of this species, as reported previously (Cox and John-Alder, 2005).

2.3. Functional studies with dispersed adrenal cells

At necropsy, any castrated lizards exhibiting remnants of gonadal tissue were eliminated from the studies. For each treatment group, six cell incubations were analyzed (duplicate incubations from each of three lizards per treatment group). Dispersed adrenal cells were prepared from the excised adrenal glands using enzymatic dispersal and partial purification procedures described in detail previously (Carsia and John-Alder, 2003). Since adrenal steroidogenic cells (hereafter referred to as adrenocortical cells) could be easily distinguished from other cell types in a hemacytometer (Carsia and John-Alder, 2003), the final concentrations of cells in the incubations were based only on the concentration of

adrenocortical cells present in the stock suspension. Final cell suspensions (2.5×10^3–1×10^4 adrenocortical cells/ml) were incubated in 12×75 mm polypropylene culture tubes with a progressive series of concentrations of hormonal peptides, for 3 h at 34.5° in a shaking water bath. The incubation medium was Krebs–Ringer–HEPES buffer [24.2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 118.5 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl2, 1.20 mM KH2PO4, 1.20 mM MgSO4, 20 mM glucose, pH 7.5] containing bovine serum albumin (5 mg/ml; KRHGB). Incubation volumes were 100–150 μl (90% of the total incubation volume was cell suspension, 10% was a solution containing a specific concentration of ACTH [rat ACTH-(1–39)] (Bachem/Peninsula Laboratories, Inc., San Carlos, CA). Dilutions of frozen stock concentrations of ACTH (0.1 mM) were carried out in the incubation medium. Final concentrations of ACTH in the cell incubations are indicated in the figures.

In each experiment, at least 94% of the cells were viable after incubation, as indicated by trypan blue dye exclusion. The amount of stored corticosteroids in lizard adrenocortical cells is small compared to production amounts (Carsia and John-Alder, 2003). Thus, after the incubation period, samples (cells and incubation medium) were frozen without separation (−30 °C) until appropriate radioimmunoassay for progesterone, corticosterone and aldosterone.

2.4. Radioimmunoassay for progesterone, corticosterone and aldosterone

Prior to radioimmunoassay, frozen incubations were rapidly thawed in a warm water bath (~45 °C) for 5 min, cooled to room temperature and then thoroughly vortex-mixed. Corticosterone (B), aldosterone (ALDO) and progesterone (P_4: laboratory studies only) were measured directly without extraction in cell incubations using highly specific, commercially available antibody coated culture tubes (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA). Because we performed direct assays of cell incubates, quantification of hormone recovery was not done (as it would be required in a procedure involving extraction). However, standard hormone concentrations were "spiked" into aliquots of a pooled charcoal-stripped, cell incubate to control for possible discrepancies between "apparent" versus "actual" concentrations of hormones in assayed incubates. We did not perform tests of parallelism in the present experiments but have done so previously to validate the use of this assay protocol in our laboratory. Radioimmunoassays were performed with standard curves derived from stock concentrations of pure steroids (Steraloids Inc., Wilton, NH) serially diluted in KRHGB. Cross-reactivities between *B* and ALDO were 0.002% (ALDO assay) and 0.2% (*B* assay), respectively. *B* cross-reactivity in the P_4 radioimmunoassay and vice versa were 0.9% and 0.7%, respectively. P_4 cross-reactivity in the ALDO radioimmunoassay was 0.007%. As little as 20 pg P_4, 5 ng *B* and 16 pg ALDO per milliliter incubation were detected as determined by the assay analysis program as the lowest concentration of "cold" steroid that caused statistically significant displacement of radiolabel. Radioimmunoassay of reference pooled cell incubations performed with each radioimmunoassay showed within- and between-assay coefficients of variation of 4.8% and 9.7%, respectively.

2.5. Data analysis

Steroidogenic dose–response curves were fitted using a "single-site" four-parameter logistic equation model that exists as a computer program (ALLFIT). This program, its "User Guide", and the original paper describing its development (De Lean et al., 1978) are freely available as downloads at the following NIH website: http://abs.cit.nih.gov/allfit/. ALLFIT simultaneously analyzes a family of dose–response curves by applying the following four-parameter logistic equation:

$$Y = (A - D)/(1 + (X/C)^B) + D$$

where *X* and *Y* are the dose and response, respectively, *A* = expected maximal response, *D* = minimal response, *B* = slope factor, and *C* = 50% response (EC_{50}) midway between *A* and *D*. The adequacy of this logistic equation for fitting sigmoidal dose–response curves has been recognized and advocated (ALLFIT User Guide, http://abs.cit.nih.gov/allfit/). ALLFIT provides estimates of the four logistic parameters together with their approximate standard errors, which serve to index the accuracy of estimates but do not provide exact confidence limits. We used ALLFIT to estimate and statistically analyze (*F*-test and *p* values) basal and maximal ACTH-induced rates of steroid production and the 50%-effective stimulatory concentration (EC_{50}) values of ACTH. These EC_{50} values are an indicator of cellular sensitivity to ACTH: the greater the EC_{50} value, the more ACTH it takes to achieve half-maximal steroid production, and thus the lower the cellular sensitivity to ACTH. For each treatment of each species, all dose–response data were entered from duplicate incubations of preparations of adrenocortical cells from each of three lizards. We then used ALLFIT to provide statistical tests of the hypotheses that two or more curves (e.g., curves for the three treatments within *S. undulatus*) share a common parameter value by forcing the curves to share this parameter and then testing how this constraint affects several indicators of "goodness of fit". Differences were deemed significant at $P < 0.05$. We did not develop a separate estimate of each parameter of a dose–response curve for each incubation or even for each lizard. Instead, we relied on the iterative logistic curve-fitting procedure encapsulated in ALLFIT for these estimates and their statistical analysis. As described in the User Guide (http://abs.cit.nih.gov/allfit/), ALLFIT performs the residual variance test and the runs test to provide indices of goodness of fit for individual dose response curves. From the User Guide (http://abs.cit.nih.gov/allfit/): the "total number of degrees of freedom is decomposed into individual values for each curve. The approximate number of degrees of freedom for each curve is calculated by subtracting from the number of data points the effective number of parameters fitted." We do not report *F* statistics and associated degrees of freedom because their interpretation is less straightforward than if we had used ANOVA followed by a post hoc test to compare estimated parameters among treatments. Instead, we simply report associated *p* values as provided by ALLFIT.

Because most responses appeared to be polyphasic, we subsequently performed an iterative non-linear fit analysis (Bevington, 1969) of the relationship between the ACTH concentration and steroid production rates. The maximum number of ACTH-responsive phases (*n*) was determined by a forward, stepwise procedure, starting with one phase and increasing the number of phases until the goodness of fit [as determined by the *F*-test (Zar, 1996)] was not improved (at $P < 0.05$). As with ALLFIT, this program generated parameter estimates (e.g., phase-specific EC_{50} values and phase-specific maximal steroid production rates) and allowed the sharing of parameter estimates between treatment dose–response curves and their inclusion in goodness of fit determinations. The outcome of this fit analysis was interpreted as evidence of multiple ACTH-responsive phases additively contributing to the maximal response of the dose–response curves.

3. Results

3.1. Field experiment with S. undulatus males

Adrenocortical cells derived from male *S. undulatus* secreted corticosterone (B) and aldosterone (ALDO) in response to ACTH

in a dose-dependent manner (Fig. 1). Long-term (441 days) orchiectomy (ORCHX) led to a small (i.e., 17%) decrease in basal *B* production but had no effect on the maximal rate of ACTH-induced *B* production (Table 1). In contrast, basal and maximal ACTH-induced ALDO production were 47% and 73% greater in ORCHX than CON cells.

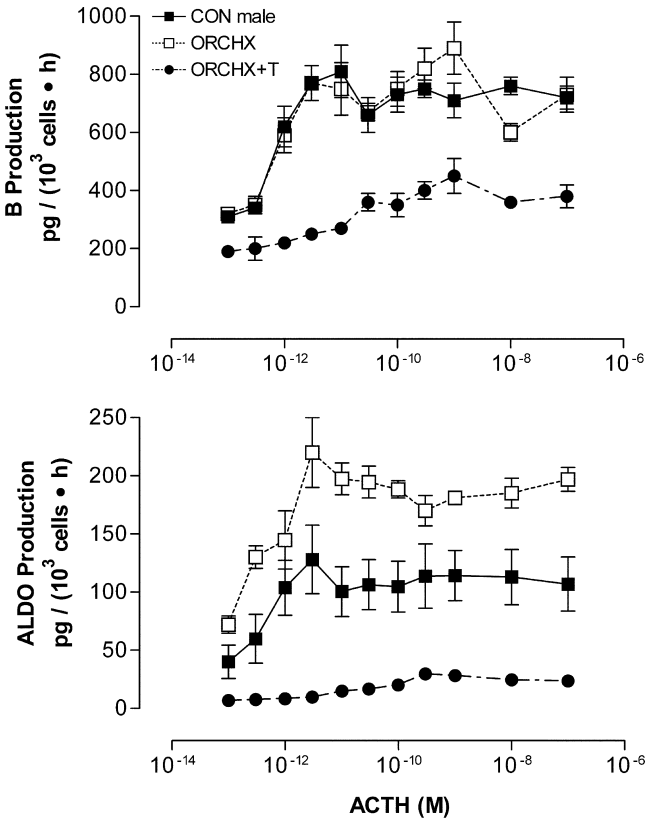

Fig. 1. Adrenocorticotropin (ACTH)-induced corticosterone (B) and aldosterone (ALDO) production rates by adrenocortical cells prepared from field-active *S. undulatus* males. Cells (2.5×10^3 cells/ml) derived from the adrenal glands of individual lizards were incubated with the indicated concentration of ACTH for 3 h at 34.5 °C. Each symbol represents the mean ± SE of values from six cell incubations (duplicate incubations of cells derived from each of three lizards). (As described under Data Analysis in Section 2, ALLFIT computes approximate standard errors.) CON, cells from intact males with empty implants; ORCHX, cells from orchiectomized male with empty implants, ORCHX + T, cells from orchiectomized males with implants containing 300 μg crystalline testosterone.

Testosterone replacement greatly suppressed cellular steroid production (Fig. 1). Basal production of *B* and ALDO were decreased 41% and 84% in ORCHX + T compared to CON cells, and maximal ACTH-induced rates of *B* and ALDO production were decreased 48% and 77%, respectively (Table 1).

Cellular sensitivity to ACTH was not affected by orchiectomy alone but was dramatically decreased by testosterone replacement, as indicated by the right shift of the ACTH dose–response curves (Fig. 1) and the increased ACTH EC_{50} values (Table 2). Whereas the ACTH EC_{50} values of CON and ORCHX cells were similar, the ACTH EC_{50} values of ORCHX + T cells for *B* and ALDO production were, respectively, 8 and 34 times those of CON cells, indicating a dramatic drop in cellular sensitivity to ACTH in response to exogenous testosterone to 13% and 3% that of CON cells.

3.2. Laboratory experiment with S. jarrovii males

Cells derived from *S. jarrovii* responded to ACTH in a dose-dependent manner (Fig. 2) similar to those of *S. undulatus*. However, short-term (6 weeks) effects of orchiectomy and testosterone replacement on adrenocortical cells derived from laboratory-housed *S. jarrovii* were different from the effects of long-term treatment in field-active *S. undulatus* (Table 3). Orchiectomy led to a small (i.e., 20%) increase in basal *B* production but had no effect on basal rates of P_4 and ALDO production or on the maximal rate of ACTH-induced *B* production. However, maximal P_4 and ALDO production in ORCHX cells were, respectively, 41% less and 57% greater than those of CON cells.

Testosterone replacement had a mixed influence on adrenal steroid production (Table 1). Basal *B* production was increased only slightly (i.e., 18%) in ORCHX + T compared to CON, and basal ALDO production was unchanged. However, the basal rate of P_4 production in ORCHX + T cells was 118% greater than that of CON cells. Maximal rates of ACTH-induced P_4 and *B* production did not differ between CON and ORCHX + T cells, indicating that testosterone replacement reversed the effect of orchiectomy on P_4 production. Maximal ALDO production was 36% greater in ORCHX + T compared to CON, indicating only a partial reversal of the effect of orchiectomy.

Orchiectomy decreased the sensitivity of *S. jarrovii* cells to ACTH. Overall, the ACTH EC_{50} values of ORCHX cells for steroid production rates were about 10 times those of CON cells (Table 2), indicating a substantial decrease in sensitivity to ACTH. Testosterone replacement tended to counteract the decrease in cellular sensitivity caused by orchiectomy, but this effect was modest and attained statistical significance only for P_4 production (Table 2).

Table 1
Basal and maximal ACTH-induced rates of steroid production in dispersed adrenocortical cells isolated from *Sceloporus* lizards [pg/(10^3cells h)]

Species	Treatment	P_4		B		ALDO	
		Basal	Maximal	Basal	Maximal	Basal	Maximal
Sceloporus undulatus	CON	NA	NA	292.4 ± 33.5[a]	744.0 ± 53.8[a]	38.2 ± 8.1[a]	111.0 ± 23.8[a]
	ORCHX	NA	NA	241.6 ± 12.7[b]	752.8 ± 61.1[a]	56.1 ± 2.5[b]	191.7 ± 13.3[b]
	ORCHX + T	NA	NA	171.3 ± 39.8[c]	383.6 ± 36.7[b]	6.1 ± 2.2[c]	25.3 ± 3.3[c]
Sceloporus jarrovii	CON	11.7 ± 1.8[a]	154.8 ± 22.0[a]	212.5 ± 1.8[a]	1607.1 ± 245.7[a]	7.9 ± 1.5[a]	33.1 ± 5.1[a]
	ORCHX	15.6 ± 3.4[a]	92.0 ± 6.6[b]	255.2 ± 36.8[b]	1435.7 ± 206.8[a]	8.2 ± 1.6[a]	51.8 ± 10.4[b]
	ORCHX + T	25.5 ± 3.8[b]	151.9 ± 14.9[a]	252.9 ± 24.5[b]	1737.0 ± 298.8[a]	9.0 ± 1.9[a]	45.4 ± 9.6[a,b]
Sceloporus virgatus	FEM	31.0 ± 0.8[a]	782.6 ± 22.8[a]	90.7 ± 3.3[a]	770.2 ± 21.4[a]	8.1 ± 0.4[a]	47.9 ± 1.4[a]
	OVX	17.5 ± 0.7[b]	664.5 ± 20.8[b]	95.1 ± 4.0[a]	758.4 ± 29.3[a]	8.5 ± 0.3[a]	46.7 ± 1.6[a]
	MALE	19.3 ± 0.9[b]	502.7 ± 16.2[c]	70.8 ± 9.1[b]	628.7 ± 26.6[b]	6.9 ± 0.1[b]	24.3 ± 0.7[b]

Note. The data of steroid production rates depicted in the figures were analyzed by the ALLFIT computer program that only analyzes data based on a single response phase. Each value represents the mean ± SE determined by the program.
Values with different superscripts are significantly different between groups within species for a particular steroid production rate at $p < 0.05$.
B = corticosterone; ALDO = aldosterone; CON = sham-operated intact male with placebo implant; ORCHX = surgically orchiectomized male with placebo implant; ORCHX + T = surgically orchiectomized male with testosterone implant; FEM = intact female; MALE = intact male.

Table 2
Half-maximal stimulatory concentration (EC_{50}; M) of ACTH for progesterone, corticosterone and aldosterone production rates of dispersed adrenocortical cells derived from *Sceloporus* lizards

Species	Treatment	EC50 for P4	EC_{50} for B	EC_{50} for ALDO
Sceloporus undulatus	CON	NA	$(7.48 \pm 0.82) \times 10^{-13a}$	$(4.40 \pm 0.87) \times 10^{-13a}$
	ORCHX	NA	$(6.01 \pm 0.51) \times 10^{-13a}$	$(3.92 \pm 0.31) \times 10^{-13a}$
	ORCHX + T	NA	$(5.43 \pm 0.38) \times 10^{-12b}$	$(1.42 \pm 0.24) \times 10^{-11b}$
Sceloporus jarrovii	CON	$(4.06 \pm 0.63) \times 10^{-11a}$	$(5.62 \pm 0.93) \times 10^{-11a}$	$(4.65 \pm 0.86) \times 10^{-11a}$
	ORCHX	$(5.18 \pm 1.14) \times 10^{-10b}$	$(4.27 \pm 0.61) \times 10^{-10b}$	$(5.43 \pm 1.09) \times 10^{-10b}$
	ORCHX + T	$(3.24 \pm 0.48) \times 10^{-10c}$	$(2.98 \pm 0.51) \times 10^{-10*}$	$(4.49 \pm 0.95) \times 10^{-10b}$
Sceloporus virgatus	FEM	$(1.47 \pm 0.39) \times 10^{-10a}$	$(7.18 \pm 2.15) \times 10^{-12a}$	$(5.93 \pm 0.83) \times 10^{-11a}$
	OVX	$(6.51 \pm 1.63) \times 10^{-10b}$	$(5.19 \pm 1.61) \times 10^{-11b}$	$(2.02 \pm 0.42) \times 10^{-10b}$
	MALE	$(1.13 \pm 0.36) \times 10^{-10a}$	$(2.93 \pm 1.26) \times 10^{-11b}$	$(1.07 \pm 0.14) \times 10^{-10c}$

Note. Each value represents the mean ± SE determined by the ALLFIT program (see Section 2) using data plotted in Figs. 1–3.
Superscripts denote statistically significant differences between treatments within species for a particular steroid EC_{50} at $p < 0.05$.
*Different from ORCHX at $p = 0.62$.
Abbreviations are as in Table 1.

3.3. Laboratory experiment with S. virgatus females

Unlike the previous studies with orchiectomized lizards, the ovariectomy study with *S. virgatus* did not include groups with sex steroid replacement. As in the other species, adrenocortical cells derived from *S. virgatus* responded to ACTH in a dose-dependent manner (Fig. 3). Basal and ACTH-induced maximal rates of steroid production were 15–50% greater in female cells (FEM) compared to male cells (MALE) (Table 1). Ovariectomy had little effect on rates of steroid production, affecting only P_4 production. Compared to FEM cells, basal and maximal ACTH-induced P_4 production of OVX cells were decreased 44% and 15%, respectively.

In general, female cells were 2–4 times more sensitive to ACTH than male cells, as indicated by the lower ACTH EC_{50} values for *B* and ALDO production (Table 2), but the ACTH EC_{50} values for cellular P_4 production were not different between the sexes. Cellular sensitivity to ACTH was consistently decreased by ovariectomy. Overall, ACTH EC_{50} values of OVX cells for steroid production rates were about five times those of FEM cells (Table 2), indicating that the sensitivity of FEM cells to ACTH was about five times that of OVX cells.

3.4. Evidence for consensus ACTH-responsive phases in Sceloporus lizard adrenocortical cells

Many of the steroid production curves for cells from *Sceloporus* lizards appeared biphasic or polyphasic, where inflections in the dose–response curves appeared to demarcate changes in rates of steroid production extending over more than a log order of ACTH concentrations to achieve a maximal rate of steroid production. Accordingly, the data comprising the response curves were pooled for each species and analyzed using the iterative curve-fitting procedure cited in the Section 2. Fit analysis was performed to determine the best fit to the statistically significant, maximal number of ACTH-response phases contributing to the maximal steroid production rate. The significantly best fit was attained with four composite or consensus phases ($p < 0.05$) (Table 3). None of the experimental groups expressed all phases. An interesting finding was that the number of phases differed among steroids, even within a single treatment for a single species. For example, for OVX females of *S. virgatus*, P_4 and *B* each exhibit two phases while ALDO exhibits three. Gonadectomy (orchiectomy in males; ovariectomy in females) caused a decrease in cellular sensitivity to ACTH apparently by inducing the expression of a phase with a relatively high ACTH EC_{50} value and having a proportionately large contribution to the maximal steroid production rate.

Polynomial fit analysis was also applied to the steroid production rates of field-active *S. undulatus*. However, the best fit in all treatment groups was to one ACTH-responsive phase. The results of the fit analysis yielded ACTH EC_{50} values (data not shown) that were very similar to those obtained by ALLFIT (listed in Table 2).

4. Discussion

The present study unequivocally demonstrates functional lability of adrenocortical cells in response to surgical gonadectomy with and without exogenous testosterone and reports for the first time *in vitro* cellular effects of the gonads on lizard adrenocortical function. Implantation of testosterone-loaded Silastic tubules led to physiologically realistic levels of plasma T in orchiectomized males of *S. jarrovii* and *S. undulatus*, as determined by comparisons to plasma T in unmanipulated males (Cox et al., 2005, 2006; Cox and John-Alder, 2005; John-Alder et al., unpublished data), and experimental manipulations in *S. undulatus* produced effects reminiscent of differences in adrenocortical cell function between intact females and males of the closely related *S. virgatus*. These observations support the physiological relevance of experimental responses reported here.

4.1. Rates of steroid production

Basal and maximal rates of steroid production in isolated, dispersed adrenocortical cells differed between females and males in *S. virgatus* and were highly labile in response to gonadectomy with or without T replacement in the other two species (Table 4). Basal and maximal rates of steroidogenesis in *S. virgatus* were consistently lower in males than in females, consistent with sex differences in various aspects of HPA (and HPI in fishes) function in diverse species (Handa et al., 1994; Pottinger et al., 1995; Canny et al., 1999; Stalvey, 2002; Viau, 2002). These sex differences have been attributed to effects of 11-ketotestosterone in rainbow trout (*Oncorhynchus mykiss*, Young et al., 1996) and testosterone in mammalian species (Stalvey, 2002; Viau, 2002). Furthermore, estradiol-17β elevated baseline and stress-induced ACTH and cortisol in rainbow trout (Pottinger et al., 1996), and a reduction in baseline cortisol in socially subordinate female marmoset monkeys has been attributed to suppression of ovarian reproductive hormones (Saltzman et al., 2000). Thus, available evidence supports the broad generalization that the HPG axis is somewhat hypofunctional in males relative to females (McQuillan et al., 2003). In the present study, the ineffectiveness of ovariectomy on *B* and ALDO production in female *S. virgatus* may reflect a decrease in ovarian steroidogenic activity induced by captivity, consistent with the absence of ovarian development evident upon necropsy.

Experimental effects on adrenocortical steroidogenesis in *S. undulatus* are generally consistent with differences between intact

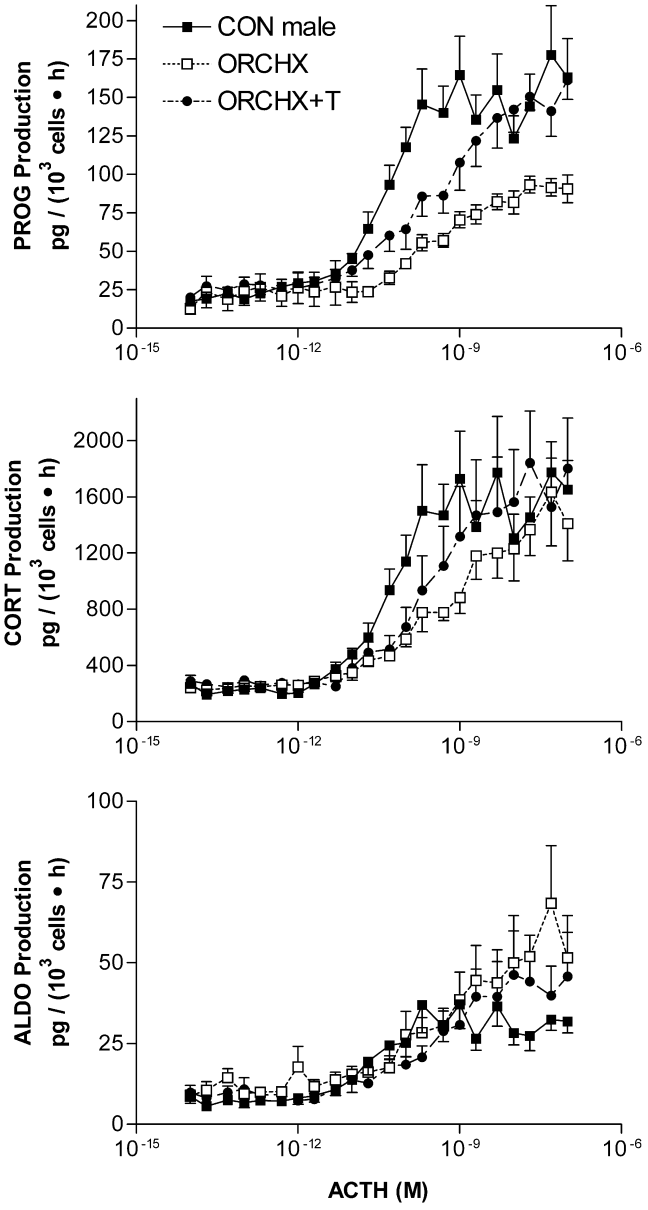

Fig. 2. Adrenocorticotropin (ACTH)-induced progesterone (P_4), corticosterone (B), and aldosterone (ALDO) production rates by adrenocortical cells prepared from laboratory-housed *S. jarrovii* males. Cells (5.0×10^3–1.0×10^4 cells/ml) derived from the adrenal glands of two lizards in each experiment were incubated with the indicated concentration of ACTH for 3 h at 34.5 °C. Each symbol represents the mean ± SE of values from six cell incubations (duplicate incubations of cells derived from each of three pairs of lizards). Treatment groups as in Fig. 1.

females and males of *S. virgatus* (Table 4). Three of four rates of steroidogenesis were either slightly or significantly higher in ORCHX than in CON, as predicted by the sex difference in *S. virgatus*, and all four reported rates of steroidogenesis were dramatically decreased by testosterone replacement. Assuming that the sexual differences in adrenal steroidogenesis that we documented in *S. virgatus* are also representative of *S. undulatus*, these results suggest that adrenocortical cells were "feminized" by orchiectomy and "masculinized" by testosterone replacement in *S. undulatus*.

By contrast, treatment effects were less prevalent in *S. jarrovii* than in *S. undulatus* and often opposite in direction to that predicted by the sex differences in *S. virgatus*. In *S. jarrovii*, for example, T replacement led to slight or significant increases in all rates of steroidogenesis with one exception (Table 4), opposite the predicted decreases in response to T. These discrepancies may be due

Table 3
Contribution (%) of a composite ACTH-response phase to the net maximal steroid production rate of dispersed adrenocortical cells from laboratory-housed lizards

Composite ACTH-response phase EC_{50} (M)	*Sceloporus virgatus*			*Sceloporus jarrovii*		
	FEM	OVX	MALE	CON	ORCHX	ORCHX + T
Progesterone						
1.45×10^{-13}	–	–	–	–	–	–
1.53×10^{-12}	18.4	17.8	19.1	–	–	–
7.57×10^{-11}	43.9	–	32.1	100.0	41.3	41.6
1.19×10^{-9}	37.7	82.2	48.8	–	58.7	58.4
Corticosterone						
1.45×10^{-13}	17.6	–	46.4	–	–	–
1.53×10^{-12}	42.2	47.1	–	–	–	–
7.57×10^{-11}	40.2	–	53.6	100.0	32.1	42.4
1.19×10^{-9}	–	52.9	–	–	67.9	57.6
Aldosterone						
1.45×10^{-13}	–	–	–	–	–	–
1.53×10^{-12}	16.5	16.7	–	–	–	–
7.57×10^{-11}	83.5	37.6	100.0	100.0	35.2	35.6
1.19×10^{-9}	–	45.7	–	–	64.8	64.4

Note. The data of steroid production rates depicted in the figures were pooled and analyzed using the polynomial equation cited in the Section 2.
Fit analysis was performed to determine the best fit to the statistically significant, maximal number of ACTH-response phases contributing to the net maximal steroid production rates.
Values are derived from the best fit which indicated four potential ACTH-response phases ($p < 0.05$).
–, indicates that the corresponding composite ACTH-response phase did not significantly contribute to the net maximal steroid production rate.
Abbreviations as in Table 1.

to differences between experimental conditions, but they may also (or alternatively) arise from differences between species in the gonadal regulation in HPA function. Within the genus of *Sceloporus* lizards, *S. jarrovii* is phylogenetically distant from *S. undulatus* and *S. virgatus*, which are very closely related (Wiens and Reeder, 1997). In addition, reproductive biology differs strikingly between *S. jarrovii* (fall breeder, live bearer) and both *S. undulatus* and *S. virgatus* (spring breeders, egg layers), and it is known, for example, that testosterone inhibits organismal growth in the latter two species while stimulating growth in *S. jarrovii* (Cox et al., 2005; Cox and John-Alder, 2005). Obviously, resolution of species discrepancies in adrenocortical cellular responses will require further comparative studies in which environmental and experimental conditions are controlled.

If testosterone were the primary regulator of adrenocortical steroidogenic capacities, we would predict from our present *in vitro* results that rates of *B* and ALDO production would vary inversely with circulating levels of testosterone. Available evidence does not support this prediction. In *S. undulatus*, basal and maximal rates of *B* production are higher at times of year marked by relatively high plasma T than at times when plasma T is very low (Carsia and John-Alder, 2003). In other words, cellular capacities for *B* production vary roughly in parallel with plasma *B* (John-Alder et al., 2002) and are greatest when plasma T is high (John-Alder et al., 1997; John-Alder et al., unpublished), consistent with the general pattern of free-living vertebrates (Romero, 2002). From the lack of correspondence between experimental *in vitro* results and previously described *in vivo* patterns, it is obviously problematic to extrapolate from *in vitro* cellular functions to *in vivo* conditions in the wild. However, it is equally clear that knowledge of lability in adrenocortical cellular functions must be taken into account to explain natural variation in glucocorticoid output of the vertebrate HPA.

Effects of ACTH on P_4 and *B* production are the same for each steroid in *S. virgatus*. Proportionately high rates of P_4 production relative to *B* in response to ACTH are also seen with adrenocortical

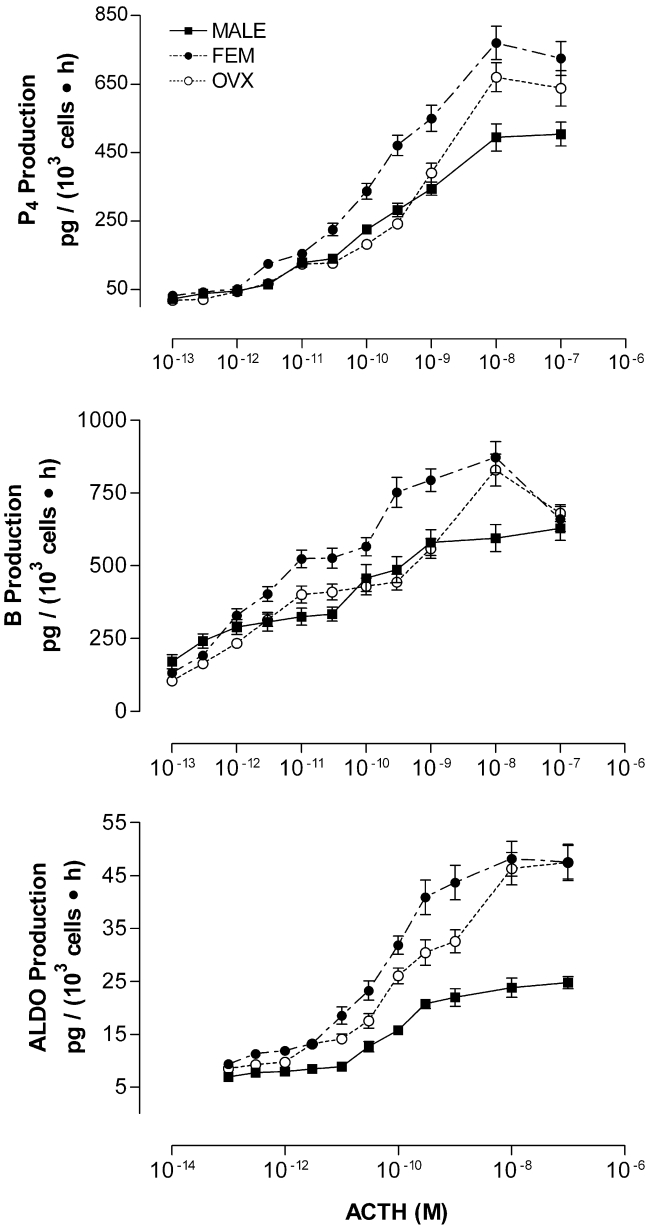

Fig. 3. Adrenocorticotropin (ACTH)-induced progesterone (P_4), corticosterone (B), and aldosterone (ALDO) production rates by adrenocortical cells prepared from laboratory-housed *S. virgatus* females and males. Cells (3.3×10^3–5.0×10^3 cells/ml) derived from the adrenal glands of two lizards in each experiment were incubated with the indicated concentration of ACTH for 3 h at 34.5 °C. Each symbol represents the mean ± SE of values from six cell incubations (duplicate incubations of cells derived from each of three pairs of lizards) from intact females (FEM), ovariectomized females (OVX) and intact males (MALE).

cells derived from *S. undulatus* (Carsia and John-Alder, 2003). However, this is not the case with cells from male *S. jarrovii*, in which P_4 production is no more than 10% that of B. These findings with adrenocortical cells derived from *Sceloporus* lizards support the notion that the adrenal gland in some lizard species may serve as an accessory source of progesterone (see Dauphin-Villemant and Xavier, 1985).

Few previous investigators have analyzed dispersed adrenocortical cells in primary culture using techniques comparable to those used in the present report, so direct comparisons with other studies are problematic. The absence of effects of orchiectomy on maximal rates of *B* production by cells from *S. jarrovii* and *S. undulatus* and the suppressive effect of T replacement on rates of *B* production by cells derived from *S. undulatus* are somewhat similar to what was seen in a comparable study with immature domestic fowl (*Gallus gallus domesticus*) (Carsia et al., 1987a,b).

Table 4
Summary of basal and maximal rates of adrenocortical steroidogenesis in *S. virgatus*, *S. undulatus*, and *S. jarrovii* in response to surgical ovariectomy or orchiectomy and testosterone replacement

	P4		B		ALDO	
	Basal	Max	Basal	Max	Basal	Max
S. virgatus						
Male	↓	↓↓	↓	↓	↓	↓
OVX	↓	↓	—	—	—	—
S. undulatus						
ORCHX	NA	NA	↓	—	↑	↑
ORCHX + T	NA	NA	↓↓	↓↓	↓↓	↓↓
S. jarrovii						
ORCHX	—	↓	↑	—	—	↑
ORCHX + T	↑	—	↑	—	—	↑

Note. Significant differences with respect to intact females of *S. virgatus* or control males of *S. undulatus* and *S. jarrovii* are indicated by *arrows*.
The relative magnitude of differences is indicated by the number of arrows.
"—" indicates no significant change.
NA = not available.
See Tables 1 and 2 for the quantitative basis of this summary table.

4.2. Sensitivity of lizard adrenocortical cells to ACTH

Adrenocortical cellular sensitivity to stimulation by ACTH was highly responsive to gonadectomy with or without T replacement, showing significant treatment responses in 13 of 16 measured cases (Table 2). Nevertheless, effects of experimental treatments on cellular sensitivity to ACTH varied with species, sex and environmental conditions. In field-active *S. undulatus*, orchiectomy alone was without effect, but T replacement in orchiectomized males drastically reduced cellular sensitivity. In some ways, these finding with field-active *S. undulatus* are similar to those with domestic fowl (*Gallus gallus domesticus*), in which orchiectomy was without effect but androgen replacement caused a dose-dependent reduction in corticosterone production (but not cellular sensitivity to ACTH) (Carsia et al., 1987a,b).

Effects of gonadectomy on cellular sensitivity to ACTH were more consistent between laboratory-housed males of *S. jarrovii* and females of *S. virgatus*. In both cases, gonadectomy decreased overall cellular sensitivity to ACTH. These findings are consistent with the decrease in cellular sensitivity to ACTH accompanying post-breeding gonadal regression in *S. undulatus* (Carsia and John-Alder, 2003). It is interesting to note that, during the breeding period of *S. undulatus*, when gonadal steroid activity is greatest, adrenocortical cell sensitivity to ACTH is greatest (Carsia and John-Alder, 2003). However, in the present study with cells derived from orchiectomized *S. undulatus* and *S. jarrovii*, testosterone replacement either drastically reduced or had little effect on cellular sensitivity to ACTH. The present study provides additional circumstantial evidence that the gonads have a non-steroidal modulating effect on adrenocortical cell function, a postulate raised in previous studies with mammalian (Kitay et al., 1966) and avian (Nagra et al., 1965; Carsia et al., 1987a,b) adrenal preparations.

4.3. Polyphasic response curves

Adrenocortical cellular responses to ACTH appear to be polyphasic, in which the different phases of steroidogenic response operate within different ranges of ACTH concentration (see Table 3). To our knowledge, polyphasic response curves have never been reported in studies with adrenocortical cells derived from any

other vertebrate species. Polynomial analysis of the pooled cellular steroidogenic responses to ACTH suggests that there are four response phases to ACTH. This suggests that the adrenal steroidogenic pathway in lizards is finely modulated, allowing the output of different ratios of steroid (B, ALDO and P_4) secretion. This modulation may occur in at least two ways. First, since the lizard adrenal gland is likely composed of functionally distinct subpopulations of cells, the reported observations may be due to a shift in the proportion of the subpopulations having different patterns of response to ACTH and different ratios of steroid species secretion. This postulate has strong support from recent work with subpopulations of adrenocortical cells prepared from the chicken (*Gallus gallus domesticus*) after dietary protein restriction (Carsia and Weber, 2000a) and the turkey (*Meleagris gallopavo*) after dietary protein restriction (Carsia and McIlroy, 1998; Carsia and Weber, 2000b) and sodium restriction (Kocsis et al., 1995). Second, if subpopulations of cells do exist in the lizard adrenal gland, the influence of gonadectomy is distributed among all subpopulations. These postulates are currently under investigation.

The physiological significance of response phases operating at very high concentrations of ACTH (the last two represent concentrations of ACTH approaching 350 and 5500 pg/ml) is unclear. Firstly, it must be remembered that these are isolated cells and a direct extrapolation to cellular responses *in vivo* cannot be made. Secondly, these seemingly non-physiological response phases may represent temporal summation of responses operating at physiological high circulating concentrations of ACTH.

4.4. Summary

The present study demonstrates functional differences between males and females and indicates that the complex role of the gonad in the regulation of adrenal steroidogenic responses at the cellular level can vary with species, sex, environment, and experimental conditions. Our opportunistic approach in the present report precludes unambiguous identification of causes of variation among species and experiments, and further experimental studies will be required to resolve present discrepancies. However, without the complexity of variables in the present studies, we would not have discovered the full scope of complexity of natural variation and experimental lability in adrenocortical cellular functional properties. Our opportunistic approach was unable to resolve the independent roles of these variables, but instead identifies them as subjects in need of further investigation. Clearly, controlled studies are needed to determine the importance of each of these variables and to establish the physiological significance of functional variation at the cellular level. Furthermore, lizards are well suited for studies of the fundamental regulation of steroidogenic pathways in adrenocortical cells isolated under different physiological conditions.

Acknowledgments

We thank Kristen Facente, Jessica Malisch, Victoriya Zilberman, and the farm staff of the School of Environmental and Biological Sciences for assistance with animal care. This research was conducted in accordance with the Arizona Game and Fish Department (permits SP 751920 and 553889), the United States Forest Service, the New Jersey Department of Environmental Protection, Division of Fish and Wildlife (permits SH 24044 and 25086), and the Rutgers University Animal Care and Facilities Committee (protocol 01-019). This project was funded by the American Museum of Natural History (Theodore Roosevelt Memorial Fund and SWRS Student Support Fund), the Graduate School-New Brunswick at Rutgers University (Pre-dissertation Travel Award), the Society for Integrative and Comparative Biology (Grant-In-Aid of Research), and the National Science Foundation (IBN 0135167).

References

Arnold, A.P., Burgoyne, P.S., 2004. Are XX and XY brain cells intrinsically different?. Trends Endocrinol. Metab. 15, 6–11.

Bevington, P.R., 1969. Data Reduction and Error Analysis for the Physical Sciences. McGraw-Hill, New York.

Canny, B.J., O'Farrell, K.A., Clarke, I.J., Tilbrook, A.J., 1999. The influence of sex and gonadectomy on the hypothalamo-pituitary-adrenal axis of the sheep. J. Endocrinol. 162, 215–225.

Carsia, R.V., Cox, R.M., McIlroy, P.J., John-Alder, H.B., 2004. Ovarian influence on adrenocortical cell function in *Sceloporus virgatus* (striped plateau lizard). Int. Comp. Biol. 44, 681. Abstract.

Carsia, R.V., Harvey, S., 2000. Adrenals. In: Whittow, G.C. (Ed.), Sturkie's Avian Physiology, fifth ed. Academic Press, New York, pp. 489–537.

Carsia, R.V., John-Alder, H.B., 2003. Seasonal alterations in adrenocortical cell function associated with stress-responsiveness and sex in the Eastern Fence Lizard (*Sceloporus undulatus*). Horm. Behav. 43, 408–420.

Carsia, R.V., McIlroy, P.J., 1998. Dietary protein restriction stress in the domestic turkey (*Meleagris gallopavo*) induces hypofunction and remodeling of adrenal steroidogenic tissue. Gen. Comp. Endocrinol. 109, 140–153.

Carsia, R.V., Morin, M.E., Rosen, H.D., Weber, H., 1987a. Ontogenic corticosteroidogenesis of the domestic fowl: response of isolated adrenocortical cells. Proc. Soc. Exp. Biol. Med. 184, 436–445.

Carsia, R.V., Reisch, N.M., Fennel, M.J., Weber, H., 1987b. Adrenocortical function of the domestic fowl: effects of orchiectomy and androgen replacement. Proc. Soc. Exp. Biol. Med. 185, 223–232.

Carsia, R.V., Weber, H., 2000a. Dietary protein restriction stress in the domestic chicken (*Gallus gallus domesticus*) induces remodeling of adrenal steroidogenic tissue that supports hyperfunction. Gen. Comp. Endocrinol. 120, 99–107.

Carsia, R.V., Weber, H., 2000b. Remodeling of turkey adrenal steroidogenic tissue induced by dietary protein restriction: the potential role of cell death. Gen. Comp. Endocrinol. 118, 471–479.

Carsia, R.V., Weber, H., Satterlee, D.G., 1988. Steroidogenic properties of isolated adrenocortical cells from Japanese quail selected for high serum corticosterone response to immobilization. Dom. Anim. Endocrinol. 5, 231–240.

Chester Jones, I., 1957. The Adrenal Cortex. Cambridge University Press, London. pp. 203–205.

Cox, R.M., 2006. A test of the reproductive cost hypothesis for sexual size dimorphism in Yarrow's spiny lizard *Sceloporus jarrovii*. J. Anim. Ecol. 75, 1361–1369.

Cox, R.M., John-Alder, H.B., 2005. Testosterone has opposite effects on male growth in lizards (*Sceloporus* spp.) with opposite patterns of sexual size dimorphism. J. Exp. Biol. 208, 4679–4687.

Cox, R.M., Skelly, S.L., John-Alder, H.B., 2005. Testosterone inhibits growth in juvenile male eastern fence lizards (*Sceloporus undulatus*): implications for energy allocation and sexual size dimorphism. Physiol. Biochem. Zool. 78, 531–545.

Cox, R.M., Zilberman, V., John-Alder, H.B., 2006. Environmental sensitivity of sexual size dimorphism: laboratory common garden removes effects of sex and castration on lizard growth. Func. Ecol. 20, 880–888.

Dallman, M.F., Akana, S.F., Scribner, K.A., Bradbury, M.J., Walker, C.-D., Strack, A.M., Cascio, C.S., 1992. Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. J. Neuroendocrinol. 4, 517–526.

Dauphin-Villemant, C., Leboulenger, F., Vaudry, H., 1990. Adrenal activity in the female lizard *Lacerta vivipara* Jacquin during artificial hibernation. Gen. Comp. Endocrinol. 79, 201–204.

Dauphin-Villemant, C., Xavier, F., 1985. *In vitro* steroid biosynthesis by the adrenal gland of the female *Lacerta vivipara* Jacquin: the metabolism of exogenous precursors. Gen. Comp. Endocrinol. 58, 1–9.

De Lean, A., Munson, P.J., Rodbard, D., 1978. Simultaneous analysis of families or sigmoidal curves: application to bioassay, radioligand assay and physiological dose–response curves. Am. J. Physiol. 235, E97–E102.

Grassman, M., Hess, D.L., 1992. Sex differences in adrenal function in the lizard *Cnemidophorus sexlineatus*: II. Responses to acute stress in the laboratory. J. Exp. Zool. 264, 183–188.

Handa, R.J., Burgess, L.H., Kerr, J.E., O'Keefe, J.A., 1994. Gonadal steroid receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Horm. Behav. 28, 464–476.

John-Alder, H., Seitz, L., Haenel, G., 1997. Ecological endocrinology of reptiles: hormonal causes and correlates of variation in exercise endurance in lizards. In: Kawashima, S., Kikuyama, S. (Eds.), Advances in Comparative Endocrinology. Proceedings of the XIIIth International Congress of Comparative Endocrinology, Yokohama, Japan. Monduzzi Editore, Bologna, Italy, pp. 1661–1667.

John-Alder, H., Carsia, R., Smith, L., Haenel, G., 2002. Seasonal and sexual variation in plasma corticosterone and adrenocortical cell function in eastern fence lizards (*Sceloporus undulatus*). In: Proceedings of the 21st Conference of European Comparative Endocrinologists, Bonn, Germany, pp. 141–146.

Kar, A.B., 1947. The adrenal cortex testicular relations in the fowl: the effects of castration and replacement therapy on the adrenal cortex. Anat. Rec. 99, 177–197.

Kitay, J.I., Coyne, M.D., Nelson, R., Newsom, W., 1966. Relation of the testis to adrenal enzyme activity and adrenal corticosterone production in the rat. Endocrinology 78, 1061–1066.

Kocsis, J.F., Carsia, R.V., 1989. Steroidogenic properties of isolated Turkey adrenocortical cells. Dom. Anim. Endocrinol. 6, 121–131.

Kocsis, J.F., Lamm, E.T., McIlroy, P.J., Scanes, C.G., Carsia, R.V., 1995. Evidence for functionally distinct subpopulations of steroidogenic cells in the domestic turkey (*Meleagris gallopavo*) adrenal gland. Gen. Comp. Endocrinol. 98, 57–72.

Malendowicz, L.K., 1994. Cytophysiology of the Mammalian Adrenal Cortex as Related to Sex, Gonadectomy and Gonadal Hormones. Wydawnictwo Poznańskiego Towarzystwa Przyjaciół Nauk, Poznań, Poland.

Manzo, C., Zerani, M., Gobbetti, A., Di Fiore, M.M., Angelini, F., 1994. Is corticosterone involved in the reproductive processes of the male lizard, *Podarcis sicula sicula*? Horm. Behav. 28, 117–129.

McQuillan, H.J., Lokman, P.M., Young, G., 2003. Effects of sex steroids, sex, and sexual maturity on cortisol production: an *in vitro* comparison of Chinook salmon and rainbow trout interrenals. Gen. Comp. Endocrinol. 133, 154–163.

Nagra, C.L., Sauers, A.K., Wittmaier, H.N., 1965. Effect of testosterone, progestagens, and metopirone on adrenal activity in cockerels. Gen. Comp. Endocrinol. 5, 69–73.

Pottinger, T.G., Balm, P.H.M., Pickering, A.D., 1995. Sexual maturity modifies the responsiveness of the pituitary–interrenal axis to stress in male rainbow trout. Gen. Comp. Endocrinol. 98, 311–320.

Pottinger, T.G., Carrick, T.R., Hughes, S.E., Balm, P.H.M., 1996. Testosterone, 11-ketotestosterone, and estradiol-17β modify baseline and stress-induced interregnal and corticotropic activity in trout. Gen. Comp. Endocrinol. 104, 284–295.

Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol. Reprod. 45, 523–532.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen. Comp. Endocrinol. 128, 1–24.

Saltzman, W., Prudom, S.L., Schultz-Darken, N.J., Abbott, D.H., 2000. Reduced adrenocortical responsiveness to adrenocorticotropic hormone (ACTH) in socially subordinate female marmoset monkeys. Psychoneuroendocrinology 25, 463–477.

Sapolosky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating, permissive, suppressive, stimulatory, and adaptive actions. Endocr. Rev. 21, 55–89.

Stalvey, J.R.D., 2002. Inhibition of 3β-hydroxysteroid dehydrogenase–isomerase in mouse adrenal cells: a direct effect of testosterone. Steroids 67, 721–731.

Tilbrook, A.J., Turner, A.I., Clarke, I.J., 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Rev. Reprod. 5, 105–113.

Viau, V., 2002. Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. J. Neuroendocrinol. 14, 506–513.

Wiens, J.J., Reeder, T.W., 1997. Phylogeny of the spiny lizards (*Sceloporus*) based on molecular and morphological evidence. Herpetol. Monogr. 11, 1–101.

Young, G., Thorarensen, H., Davie, P.S., 1996. 11-Ketotestosterone suppresses interrenal activity in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 103, 301–307.

Zar, J.H., 1996. Biostatistical Analysis, third edition. Prentice-Hall, Upper Saddle River.