

## Effects of food restriction on steroidogenesis in dispersed adrenocortical cells from Yarrow's Spiny Lizard (*Sceloporus jarrovi*)

Rocco V. Carsia<sup>a</sup>, Patrick J. McIlroy<sup>b</sup>, Robert M. Cox<sup>c</sup>, Michele Barrett<sup>d</sup>, Henry B. John-Alder<sup>e,\*</sup>

<sup>a</sup> Department of Cell Biology, University of Medicine and Dentistry of New Jersey, School of Osteopathic Medicine, Stratford, NJ 08084, USA

<sup>b</sup> Department of Biology, Camden College of Arts and Sciences, Rutgers University, Camden, NJ 08102, USA

<sup>c</sup> Department of Biology, University of Virginia, Charlottesville, VA 22904, USA

<sup>d</sup> Department of Animal Sciences, Rutgers University, New Brunswick, NJ 08901, USA

<sup>e</sup> Department of Ecology, Evolution, and Natural Resources, Rutgers University, New Brunswick, NJ 08901, USA

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### ABSTRACT

Changes in energy balance can lead to functional alterations at all levels of the hypothalamic–pituitary–adrenal (HPA) axis. However, relatively little is known about how energy balance affects functional properties of adrenocortical cells themselves. We investigated effects of restricted food intake on sensitivity to ACTH and rates of steroidogenesis in adrenocortical cells isolated from growing female and male Yarrow's Spiny Lizards (*Sceloporus jarrovi*). At the end of the feeding regimen, we assayed acute (3 h) progesterone (P<sub>4</sub>), corticosterone (B), and aldosterone (ALDO) production in response to ACTH in dispersed adrenocortical cells. Food restriction depressed growth rate by about 50% in both males and females but did not alter baseline plasma B measured at 10 weeks in either sex. At the cellular level, food restriction had the following effects: (1) increased basal B production in both sexes and basal ALDO production in males, (2) increased net maximal rates of production of P<sub>4</sub>, B, and ALDO in response to ACTH, and (3) no overall effect on adrenocortical cellular sensitivity to ACTH. There were modest sex differences: overall rates of P<sub>4</sub> production were 46% greater in cells from females than from males, and in response to food restriction, the net maximal rate of ALDO production was 50% greater in cells from males than from females. Our results demonstrate that food restriction in *S. jarrovi* increases adrenocortical cellular rates of steroid production without affecting overall cellular sensitivity to ACTH.

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### 1. Introduction

Adrenocortical function plays a central role in vertebrate intermediate metabolism, and nutrition has a profound impact on the hypothalamic–pituitary–adrenal (HPA) axis in both mammalian and non-mammalian species. In laboratory rats (*Rattus norvegicus*), food intake has been shown experimentally to influence circadian periodicity in plasma corticosterone [35], and a similar response may occur in marine iguanas (*Amblyrhynchus cristatus*) in association with foraging [54]. Furthermore, in free-living animals, requirements for energy mobilization have been invoked as the reason for seasonal variation in glucocorticoid output of the HPA axis [42]. Studies on the laboratory rat indicate that the activity of the HPA axis is part of a larger homeostatic system that regulates caloric flow [1,21,22,23,32,41,49,57]. In the guinea pig (*Cavia porcellus*), even brief periods of maternal caloric restriction can imprint abnormal HPA axial function that is expressed in adult off-

spring [36]. Functional lability involves both the proximal portion of the HPA axis (hypothalamus and pituitary) as well as the distal portion (adrenal cortex). With regard to the adrenal cortex itself, the increase in adrenocortical steroidogenesis by caloric restriction is thought to be mediated in part by alterations in circulating leptin and the activity of adrenal innervation [21].

In birds, evidence suggests an interaction between hormonal regulation of energy (fat) stores and the regulation of the HPA axis. Furthermore, in controlled studies, food restriction of sufficient duration typically increases plasma concentrations of corticosterone (B) [6,37,43,53]. Less information is available for ectothermic vertebrates, but the existing findings on the impact of food restriction on interrenal/adrenal steroidogenic function are varied. In tadpoles, for example, steroidogenic functions of the hypothalamic–pituitary–interrenal (HPI) axis are increased by food restriction, reflecting the need to mobilize tail energy stores for development and metamorphosis [19,30]. However, in juvenile frogs, food restriction does not appear to alter interrenal steroidogenic function because, without sizable energy stores, it is thought that increases in circulating corticosteroids would be deleterious to energy balance [19,20]. By contrast, food restriction in fish appears to increase interrenal steroidogenesis [38].

\* Corresponding author. Address: Department of Ecology, Evolution, and Natural Resources, Rutgers University, 14 College Farm Road, New Brunswick, NJ 08901. Fax: +1 732 932 8746.

E-mail address: [henry@aesop.rutgers.edu](mailto:henry@aesop.rutgers.edu) (H.B. John-Alder).

Most previous research has focused on the proximal portion of the HPA axis (i.e., hypothalamic–pituitary responses). By contrast, effects of food restriction on the adrenal gland itself have largely been neglected [21]. Studies on species of domestic fowl indicate that dietary protein restriction increases adrenal steroidogenic function at the cellular level in chickens (*Gallus gallus domesticus*) [11,13,14,40,51] and has the opposite effect in turkeys (*Meleagris gallopavo*) [8,12]. It follows that investigations of lability in adrenocortical cellular functions are likely to be informative and variable in other species. While investigations exploring the impact of caloric restriction on avian species are numerous, similar studies on non-avian reptiles – including the species-rich and ecologically diverse Squamata (lizards and snakes) – are lacking. Such information can help to bridge the gap in knowledge between non-mammalian endothermic vertebrates (i.e., birds) and ectothermic vertebrates (non-avian reptiles, amphibians and fish), especially since birds are evolutionarily derived reptiles. Furthermore, an understanding of how the reptilian HPA axis is modulated by stress, including nutritional stressors, will help to clarify comparative questions of functional variation among species [42]. Representative species of Squamata can be particularly opportune for this purpose because of their ecological diversity.

In the present contribution, we report the influence of food restriction on adrenal steroidogenic (adrenocortical) cell function in Yarrov's Spiny Lizard (*Sceloporus jarrovii*). We used dispersed adrenocortical cells harvested from lizards that had been involved in an investigation of the influence of food restriction on energy allocation to growth and body composition in lizards [17]. Dispersed adrenocortical cell preparations have been used to help characterized adrenal functional lability in response to stress in avian species [8,10,11,12,14,40,51], and we have previously validated this approach to investigate components of adrenal stress response in lizards [7].

In our previous study of seasonal variation in adrenocortical cellular function in field-active *Sceloporus undulatus* [7], we reported substantial decreases in cellular sensitivity to ACTH and maximal rates of ACTH-induced B production from the breeding season in early spring to the post-breeding period of mid-summer, accompanied by a deterioration in body condition. These results suggest that food restriction may cause reductions in steroidogenic capacities and sensitivity to ACTH, a prediction that we test in the present study using adrenocortical cells from *S. jarrovii*. However, any such prediction is rather tenuous, given differences between species of lizards, their natural habitats, and experimental conditions. Furthermore, adrenocortical responses to dietary manipulation have previously been shown to differ between chickens and turkeys even under very similar experimental conditions (see above). Thus, the alternative prediction, that food restriction will cause an increase in cellular steroidogenic capacities in *S. jarrovii*, as in domesticated turkeys [8,12], is almost equally plausible based on current evidence.

As part of our assessment of cellular functions, we report  $P_4$  in addition to B and ALDO production. The emerging picture is that adrenal  $P_4$  plays significant gestational [29] and behavioral roles in lizards [33,52]. In several species of lizards,  $P_4$  is a significant secretory product in *in vitro* adrenal preparations [24,25,29,31,33]. Furthermore, our previous work with dispersed adrenocortical cells from *Sceloporus* lizards has demonstrated modest to robust  $P_4$  production in response to ACTH [7,9].

## 2. Materials and methods

### 2.1. Animals

All experimental procedures were reviewed and approved by the Rutgers University Animal Care and Facilities Committee (protocol #01-019). As reported previously [17], male and female *S. jarrovii*

yearlings (2–3 months old) were collected in September, 2004 near Buena Vista Peak in the Chiricahua Mountains, Coronado National Forest, Arizona, USA (31°54'–55'N, 109°16'W). Animals were collected under permit from the Arizona Game and Fish Department (SP 553889) and housed at Rutgers under permit from the New Jersey Division of Fish and Wildlife (SH 25086). In the laboratory, lizards were held individually in plastic cages (36 × 42 × 46 cm) containing a bedding of sand and two bricks that were stacked to form a shelter and basking site. Water was always available in a shallow dish lined with aquarium gravel. Lizards were housed on a 12:12 L:D photoperiod with an incandescent light as a heat source on a 10-h thermal period. Temperatures within cages varied along a gradient from 25 to 45 °C during photophase and averaged 19 °C during scotophase. Several criteria, including plasma B, indicate that these captivity conditions after an acclimation period were not stressful [16,17,18].

Animals were acclimated to captivity for 1 week and were then assigned to one of four size-matched treatment groups: high-food males ( $n = 11$ ) and females ( $n = 8$ ), and low-food males ( $n = 10$ ) and females ( $n = 12$ ). These four groups did not differ in initial snout-vent length (SVL) ( $F_{3,37} = 0.82$ ;  $P = 0.49$ ) or body mass ( $F_{3,37} = 0.24$ ;  $P = 0.87$ ) prior to food manipulation. For 10 weeks, high-food groups were provided three crickets (*Acheta domestica*) per day, while low-food groups were provided one cricket/d. Each week, all live and dead crickets that remained in each cage were counted, and food consumption (crickets/d) for each animal was estimated by assuming that all missing crickets had been consumed. The high-food ration of three crickets per day allowed lizards to feed *ad libitum* [17] in that lizards under this feeding regimen had demonstrably not eaten all the crickets offered. Thus, groups fed three crickets per day are the unrestricted, *ad libitum*-feeding controls. Hereafter, the feeding groups are designated “*ad libitum*-fed” and “food-restricted”. Snout-vent length (mm) and body mass (g) were measured at biweekly intervals, and growth rate in SVL (mm/d) and body mass (g/d) were estimated as the slope of the linear regression of body size (SVL or mass) on elapsed time (d) for each individual lizard [16]. Growth was linear over the duration of the 70-d experimental period, as assumed by this method. Furthermore, the food-restriction group continued to grow albeit more slowly than the *ad libitum*-fed controls (see Section 3). Thus, the food-restriction regimen was not starvation.

At the conclusion of the experiment, animals were euthanized by decapitation, blood was collected, and carcasses were necropsied for adrenal glands. Blood for determination of plasma B was collected in heparinized microhematocrit capillary tubes from the open neck wound. Blood samples were centrifuged, and plasma fractions were collected in capped microfuge tubes and held at –20 °C until thawed for radioimmunoassay. Adrenal glands were immersed and stored in ice-cold basic medium (see below) containing bovine serum albumin (1 mg/ml; Fraction V; Sigma Chemical Co., St. Louis, MO) until processing for dispersed adrenal cells.

### 2.2. Preparation of dispersed lizard adrenal cells

Adrenal glands (two per lizard) were harvested from a subset of eight lizards chosen arbitrarily from each feeding group. Adrenocortical cells for incubations were prepared by pooling both adrenal glands from each of two lizards (i.e., 4 adrenal glands). Thus, a total of four independent pooled cell preparations, where each preparation contained cells from two lizards, were analyzed for each feeding group. Each pooled cell preparation was incubated in duplicate under each incubation condition, resulting in a total of eight incubations under each condition for each feeding group.

The basic medium used for adrenal tissue dissociation and dispersed cell incubation was Krebs–Ringer–HEPES buffer containing glucose [24.2 mM HEPES; (*N*-2-hydroxyethyl)piperazine-*N'*-2-eth-

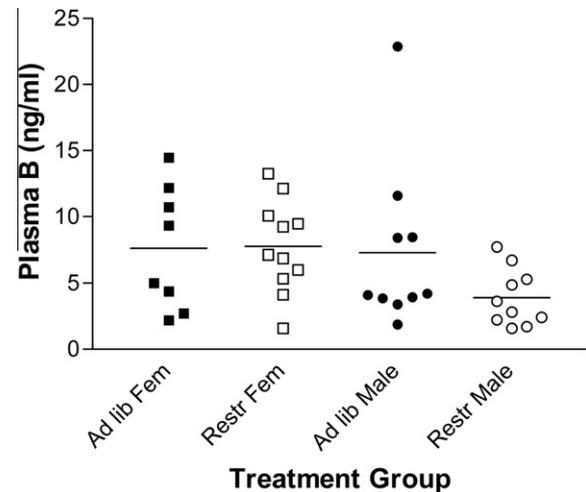
anesulfonic acid), 118.5 mM NaCl, 4.75 mM KCl, 2.54 mM  $\text{KH}_2\text{PO}_4$ , 1.20 mM  $\text{MgSO}_4$ , 20 mM glucose, pH 7.5 (KRHG)]. The dissociation medium consisted of this basic medium containing bovine serum albumin (2.5 mg/ml; Fraction V; Sigma), collagenase (5.0 mg/ml; approximately 195 U/mg; Worthington Biochemical Corp., Lakewood, NJ), lima bean trypsin inhibitor (0.1 mg/ml; Sigma), and DNase (0.05 mg/ml; Sigma). Paired adrenal glands from a single lizard were delivered in  $\sim 500 \mu\text{l}$  of dissociating medium into plastic culture dishes ( $100 \times 20 \text{ mm}$ ; Corning Glass Works, Corning, NY). Glands were finely minced with dissection scissors, the scissors were carefully rinsed into the dish with  $\sim 500 \mu\text{l}$  of dissociation medium, and an additional 3 ml of dissociation medium was added to a final dissociation medium volume of 4 ml. Gland tissue adhering to the wall of the culture dish was triturated using a 1-ml pipetting gun. The mixture was then carefully oscillated ( $\sim 90$  oscillations/min) in a reciprocal shaking water bath for  $\sim 20$ – $25 \text{ min}$  at  $34.5^\circ\text{C}$ , which approximates the preferred body temperature for this species. After an oscillation period, the mixture was carefully triturated about 30 times with a 1-ml pipet gun, and the oscillation was then repeated. These steps were repeated until adrenal tissue was completely dissociated (about three cycles over about 1 h). At the end of the dissociation, 5 ml of chilled incubation medium (KRHG containing bovine serum albumin, 5 mg/ml) were added to the mixture, and this 9-ml mixture was filtered through nylon fabric ( $55 \mu\text{m}$  mesh; No. 3-53/41; Sefar America Inc., Kansas City, MO) to remove large cell clumps. The dispersed adrenal cells in the filtrate were collected by centrifugation ( $300g$ , 18 min,  $4^\circ\text{C}$ ). The resultant pellet was resuspended in a small volume ( $0.5$ – $1.0 \text{ ml}$ ) of incubation medium for determination of adrenocortical cell concentration. Lizard adrenocortical cells have light microscopic features that are shared by adrenocortical cells isolated from diverse vertebrate species [15], and steroidogenic cells (adrenocortical cells) can be easily distinguished from adrenochromaffin cells and erythrocytes by their opaque, brownish lipid drop-laden cytoplasm [7]. Cell counts (20 replicate counts per preparation) were quantified with a hemacytometer (Improved Neubauer, 0.1 mm deep; American Optical, Southbridge, MA). In each of 4 experiments, the group with the limiting concentration of cells became the normalizing concentration for all groups. In the present study, the yield of adrenocortical cells per mg adrenal mass did not significantly vary between the treatment groups [ $72,800 \pm 15,300 \text{ cells/mg}$  (mean  $\pm$  SE)].

### 2.3. Functional studies

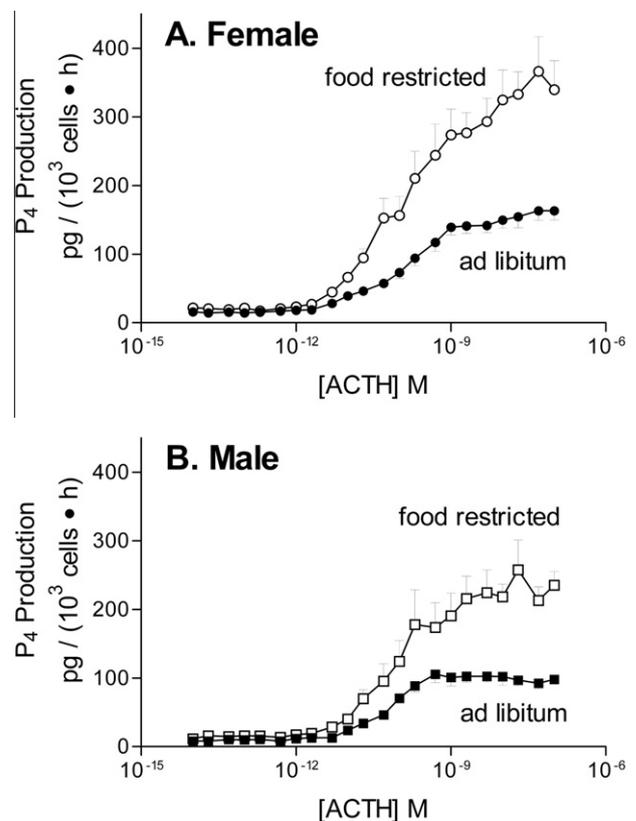
Within 3 h of necropsy, aliquots of adrenocortical cell suspensions ( $3.8 \times 10^3$  to  $10.0 \times 10^3 \text{ cells/ml}$ ) were dispensed into  $12 \times 75 \text{ mm}$  polypropylene culture tubes using a Nichiryo syringe dispenser (Model 8100; Nichiryo America Inc., Maryland Heights, MO) fitted with a disposable plastic 1.5-ml syringe for small-volume delivery ( $100 \mu\text{l}$ ). Cells were incubated with a wide range of concentrations of corticotropin (ACTH) [rat ACTH-(1–39); Bachem/Peninsula Laboratories, San Carlos, CA] for 3 h at  $34.5^\circ\text{C}$  in a shaking water bath. As indicated in Figs. 2–4, corticotropin concentrations ranged from  $10^{-14}$  to  $10^{-7} \text{ M}$ , allowing us to characterize the full range of responsiveness of dispersed cells. We have previously verified that as in adrenocortical cells derived from other species, lizard adrenocortical cells store very little steroid compared to amounts produced *de novo* [7]. Thus, total incubations were frozen ( $-30^\circ\text{C}$ ) without separation until radioimmunoassay for corticosterone (B), aldosterone (ALDO) and progesterone ( $\text{P}_4$ ).

### 2.4. Radioimmunoassay for plasma B

Plasma B was determined by radioimmunoassay following published procedures [48]. Briefly, plasma samples of measured vol-

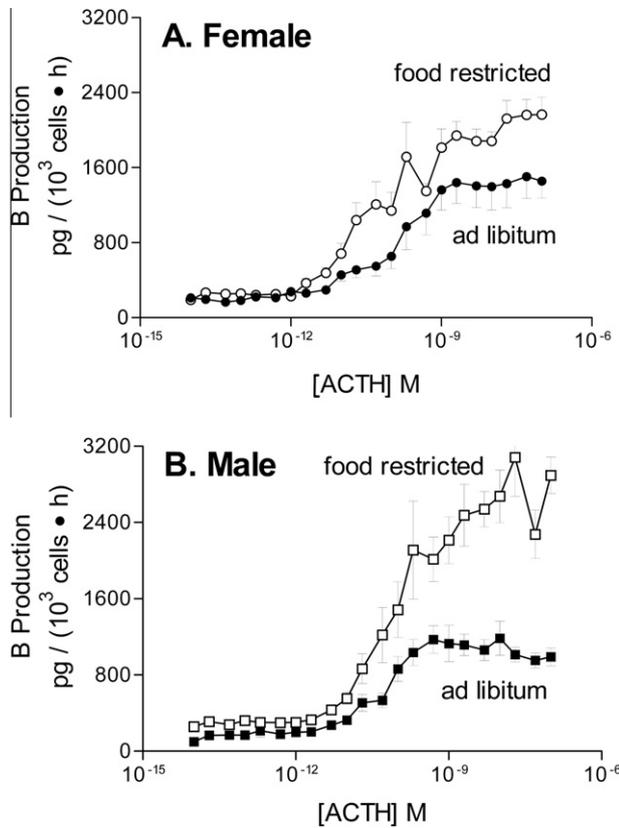


**Fig. 1.** Baseline plasma corticosterone in males and females in *ad libitum*-fed (control) and food-restricted groups. Values for all individuals in each group are illustrated. Data were analyzed by 2-way ANOVA, using treatment and sex as main effects with interaction. In the overall model, group means (horizontal lines) were statistically indistinguishable ( $F_{3,35} = 1.76$ ,  $P = 0.17$ ). Effects of treatment ( $P = 0.26$ ), sex ( $P = 0.14$ ), and the interaction term ( $P = 0.22$ ) all failed to achieve statistical significance. Ad lib = *ad libitum*-fed; Restr = food-restricted.



**Fig. 2.** Adrenocorticotropin (ACTH)-induced progesterone ( $\text{P}_4$ ) production by adrenocortical cells prepared from females and males of *S. jarrovi* maintained on 3 (*ad libitum*-fed) crickets/d or 1 (food-restricted) cricket/d.

ume (approximately  $30 \mu\text{l}$ ) were extracted twice in 2 ml of diethyl ether and dried under a stream of ultrafiltered air. Samples were reconstituted in assay buffer and were allowed to equilibrate overnight under refrigeration prior to radioimmunoassay. Corticosterone antiserum (B3–163) was purchased from Esoterix Inc. (Calabasas Hills, CA, USA). The limit of detection of the assay was 8 pg,

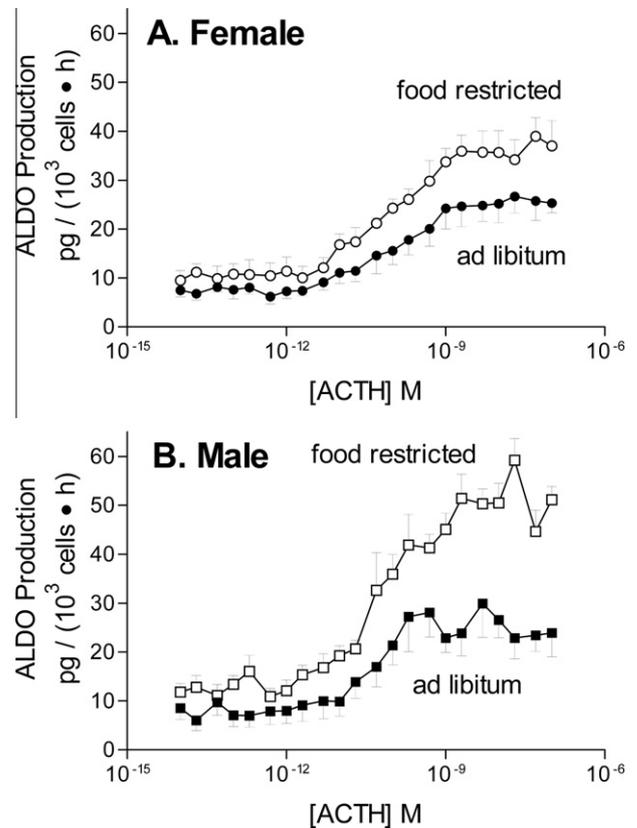


**Fig. 3.** Adrenocorticotropin (ACTH)-induced B production by adrenocortical cells prepared from females and males of *S. jarrovi* maintained on 3 (*ad libitum*-fed) crickets/d or 1 (food-restricted) cricket/d.

and the inter- and intra-assay variability were 7.5% and 8%, respectively.

### 2.5. Radioimmunoassay for B, ALDO and P<sub>4</sub>

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<sub>4</sub>) 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 would be required in a procedure involving extraction). However, standard hormone concentrations were added into aliquots of a pooled, charcoal-stripped cell incubate to control for possible discrepancies between “apparent” vs. “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 crossreactivity in the P<sub>4</sub> radioimmunoassay and *vice versa* were 0.9% and 0.7%, respectively. P<sub>4</sub> crossreactivity in the ALDO radioimmunoassay was 0.007%. As little as 20 pg P<sub>4</sub>, 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



**Fig. 4.** Adrenocorticotropin (ACTH)-induced ALDO production by adrenocortical cells prepared from females and males of *S. jarrovi* maintained on 3 (*ad libitum*-fed) or 1 (food-restricted) cricket/d.

incubations performed with each radioimmunoassay showed within- and between-assay coefficients of variation of 4.8% and 9.7%, respectively.

### 2.6. Data analysis

Effects of food restriction on growth rate (mass and length gain), body composition, and plasma testosterone have been analyzed and reported previously [17]. A preliminary analysis indicated that plasma B did not depend on body size. Subsequently, measured values of baseline plasma B were analyzed by 2-way ANOVA, using treatment and sex as main effects with interaction. ACTH produced sigmoidal curves of steroid production rates (see Figs. 2–4). However, the presence of visible inflection points and the fact that the curves extended over more than two log<sub>10</sub> orders of ACTH concentration suggested that dose–response curves were at least biphasic: that there were two or more phases of response to ACTH and that the contribution of each ACTH-response phase (or component) to the maximal rate of steroid production varied. In order to statistically analyze these phases, we performed an iterative nonlinear fit analysis [3] of the relationship between the concentration of ACTH and steroid production rates. The utility of this analysis has been previously established in our laboratory in investigations on the influence of the gonad on lizard adrenocortical cell function [9]. In brief, in this nonlinear fit analysis, the maximum number of steroid-response phases was determined by a forward, stepwise procedure where the number of phases was increased until the accuracy of the prediction was not improved according to the *F*-test with *P* < 0.05 [55]. The outcome of this fit analysis was interpreted as evidence of more than one steroid-response phase contributing to the overall maximal steroid response to ACTH. *Post*

**Table 1**  
Basal and net maximal ACTH-induced rates of steroid production (pg/10<sup>3</sup> cells h) by dispersed lizard adrenocortical cells.

Sex	Cricket ration (No./day)	P <sub>4</sub>		B		ALDO	
		Basal	Maximal	Basal	Maximal	Basal	Maximal
Female	3	15.07 ± 3.88**	132.82 ± 11.15**	203.12 ± 31.94	1194.37 ± 190.52	7.18 ± 1.35	17.77 ± 2.53
	1	19.33 ± 5.05	298.46 ± 36.78**	273.27 ± 25.85*	1988.20 ± 182.85*	10.12 ± 2.34	25.73 ± 2.97*
Male	3	8.89 ± 1.73	98.37 ± 9.10	160.29 ± 42.48	987.28 ± 113.28	7.58 ± 2.56	19.44 ± 3.60
	1	13.92 ± 3.37	215.25 ± 33.40*	282.97 ± 28.82*	2394.00 ± 290.07*	12.65 ± 2.09*	38.63 ± 3.20***

Note: Rates of steroid production illustrated in Figs. 2–4 were pooled for each hormone and statistically analyzed by an iterative polynomial equation. Each value is the mean ± SE determined by the analysis.

\* Significantly greater than the *ad libitum*-fed value of the same sex.

\*\* Significantly greater than corresponding feeding regimen value of the opposite sex.

**Table 2**  
Contribution (%) of an ACTH-response phase to the net maximal rate of steroid production in dispersed lizard adrenocortical cells.

ACTH phase component EC <sub>50</sub> (M)	Feeding regimen			
	<i>Ad libitum</i> -fed female	Food-restricted female	<i>Ad libitum</i> -fed male	Food-restricted male
<i>Progesterone</i>				
1.51 × 10 <sup>-11</sup>	22.6 ± 2.7	22.4 ± 3.9	24.5 ± 3.2	18.4 ± 4.0
1.25 × 10 <sup>-10</sup>	77.4 ± 9.2	77.6 ± 13.5	75.5 ± 10.0	81.6 ± 17.9
<i>Corticosterone</i>				
1.51 × 10 <sup>-11</sup>	26.3 ± 5.9	32.3 ± 4.2	40.3 ± 6.6	17.3 ± 3.0***
1.25 × 10 <sup>-10</sup>	73.7 ± 16.7	67.7 ± 8.8	59.7 ± 9.7	82.7 ± 14.1
<i>Aldosterone</i>				
1.51 × 10 <sup>-11</sup>	26.7 ± 5.4	31.2 ± 5.1	41.4 ± 10.8	32.0 ± 3.8
1.25 × 10 <sup>-10</sup>	73.3 ± 14.7	68.8 ± 11.2	58.6 ± 15.3	68.0 ± 8.0

Note: Rates of steroid production illustrated in Figs. 2–4 were pooled for each hormone and analyzed by an iterative polynomial equation. Fit analysis was performed to determine the best fit to the statistically significant, maximal number of ACTH-phase components contributing to the net maximal steroid production rates. Values above are from the best fit, which indicated two ACTH-phase components ( $P < 0.05$ ).

\* Significantly less than the *ad libitum*-fed value of the same sex.

\*\* Significantly less than corresponding feeding regimen value of the opposite sex.

*hoc* comparisons of these parameters of cellular response between sexes and between treatment groups were analyzed ( $F$ -test) by applying the iterative nonlinear fit analysis to the data of entire dose–response curves. Basal and ACTH-induced rates of steroid production (including maximal rates in each ACTH-response phase), the overall half-effective concentration (EC<sub>50</sub>) of ACTH, and the EC<sub>50</sub> values for the ACTH-response phases, were generated by this analysis. Differences were deemed significant at  $P < 0.05$ . The values of EC<sub>50</sub> provide an index of ACTH potency and adrenocortical cell sensitivity to ACTH. Thus, the greater the ACTH EC<sub>50</sub>, the lower the cellular sensitivity to ACTH [47].

### 3. Results

#### 3.1. Growth and baseline plasma corticosterone

During 10 weeks of the feeding regimen, food availability of three crickets per day proved to be an unrestricted, *ad libitum* ration, as reported previously [17]. Growth rate, measured as mm length gain per day, was about 0.1 mm/day in food-restricted lizards vs. about 0.2 mm/day in lizards in the *ad libitum*-fed groups ( $P < 0.001$ ) [17]. Despite these differences in food consumption and growth rates, baseline plasma B concentrations, measured after 10 weeks of controlled feeding, did not differ between sexes or feeding groups (Fig. 1).

#### 3.2. Basal steroid production rates by adrenocortical cells

Effects of the feeding regimen on basal and overall net maximal (maximal minus basal) rates of steroid production are reported in

Table 1. Food restriction had no significant effect on basal P<sub>4</sub> production. However, basal B production was significantly elevated (by 56%) in cells from food-restricted female and males. In addition, basal ALDO production was significantly elevated (by 67%) in cells from food-restricted males (but not females).

Sex differences in basal rates of steroid production were rare. Basal production of P<sub>4</sub> was significantly higher (by 70%) in *ad libitum* female than in *ad libitum* male cells, but in contrast, basal rates of B and ALDO production, irrespective of feeding regimen, did not differ between males and females.

#### 3.3. Overall net maximal rates of ACTH-induced steroid production adrenocortical cells

Food restriction had a consistent, enhancing effect on net maximal rates of steroid production in response to ACTH (Figs. 2–4; Table 1). This effect was consistently true for maximal rates of production of P<sub>4</sub>, B and ALDO in cells of both females and males. Overall, the net maximal rate of steroid production in cells from food-restricted lizards was nearly double (increase of 99.3 ± 15.2%; mean ± SE) that of cells from *ad libitum*-fed lizards.

Some sex differences in net maximal steroid production were also apparent. Overall, net maximal P<sub>4</sub> production of female cells was significantly greater (by 39%) than that of male cells. By contrast, net maximal rates of B production did not differ between males and females. However, the rate of ALDO production in cells from food-restricted males was significantly greater (by 50%) than that of cells from food-restricted females.

### 3.4. Sensitivity of adrenocortical cells to ACTH

All steroid response curves were analyzed by an iterative non-linear equation. In addition to the basal and maximal ACTH-induced rates of steroid production, this equation also generated estimates of the ACTH EC<sub>50</sub>. By confining the response data to a monophasic response to ACTH, the analysis determined no significant difference in the EC<sub>50</sub> values among the responses of the steroid species measured and no difference in the corresponding EC<sub>50</sub> values between treatment groups and between sexes. Accordingly, the collective analysis of all response data generated an overall EC<sub>50</sub> of  $(5.93 \pm 0.98) \times 10^{-11}$  M ( $\sim 270$  pg ACTH/ml). Thus, feeding regimen and sex had no influence on the overall cellular sensitivity to ACTH regardless of the steroid species analyzed.

When the data were not confined to a monophasic response to ACTH, the best fit analysis indicated the presence of two phases operating at ACTH EC<sub>50</sub> values of  $(1.51 \pm 0.25) \times 10^{-11}$  M and  $(1.25 \pm 0.21) \times 10^{-10}$  M. Here again, there were no significant differences in EC<sub>50</sub> values of either phase among the responses of the steroid species measured, and no difference in the corresponding EC<sub>50</sub> values between treatment groups and between sexes, allowing for the calculation of overall values for each phase. These EC<sub>50</sub> values correspond to ACTH concentrations of  $\sim 70$  and  $\sim 570$  pg ACTH/ml, respectively.

Further analyses were performed to determine variation in the contribution of each steroid-response phase to the overall net maximal steroidogenic response (Table 2). Cells from females were consistent in the proportional contribution of each phase to the overall steroid response, regardless of steroid and feeding treatment. Averaged over all steroids and treatments, 27% of the net steroidogenic response in cells from females is attributed to the low EC<sub>50</sub> phase, and 73% to the high EC<sub>50</sub> phase.

In males, effects of food restriction on the proportional response distribution tended to be greater than in females, but these differences failed to attain statistical significance at  $P < 0.05$  in all cases other than the high sensitivity component of the corticosterone response to ACTH (Table 2). Thus, the combined proportional distribution between the phases was not significantly different from that of females (29% and 71%, respectively). However, the possible variation is more apparent when the proportional distribution values for the different steroids are compared. For example, in cells from males, there appears to be a greater shift in distribution to the low-EC<sub>50</sub> phase vs. the high-EC<sub>50</sub> phase for ALDO and B ( $\sim 41\%$  vs. 59%, respectively). In addition, food restriction appears to have a greater effect on this distribution for these two steroids in males. This effect was significant for B: the proportional contribution of the low-EC<sub>50</sub> phase decreased from 40.3% to 17.3% ( $P < 0.05$ ), while the contribution of the high-EC<sub>50</sub> phase increased in a compensatory direction from 59.7% to 82.7% (n.s.).

## 4. Discussion

During the postbreeding season in adults of the Eastern Fence Lizard, *S. undulatus*, basal and net maximal ACTH-induced rates of B production by adrenocortical cells decrease compared to the breeding season and, at least in males, there is a substantial decrease in cellular sensitivity to ACTH [7]. These changes in adrenal steroidogenic responses at the cellular level in field-active *S. undulatus* are associated with a pronounced decrease in body condition (i.e., body mass controlled for body length), and a 10-fold reduction in plasma B in the months of July and August compared to earlier in the year [34]. Based on these results, one plausible prediction in the present study was that chronic food restriction in *S. jarrovi* would reduce adrenocortical cellular sensitivity to ACTH and decrease maximal rates of ACTH-induced B production. To the

contrary, however, food restriction increased both basal and ACTH-induced B production by dispersed adrenocortical cells (Fig. 3 and Table 1). Similar results were obtained for ALDO and P<sub>4</sub> production (Figs. 2 and 4 and Table 1). Indeed, the results of our food restriction study are very similar to those from studies of dietary protein restriction in growing cockerels (*Gallus gallus domesticus*) [6,11,13,14,40]. However, unlike the changes seen in chicken adrenocortical cells, in which there was an increase in overall cellular sensitivity to ACTH, there was no change in overall lizard adrenocortical cell sensitivity to ACTH.

Food restriction did not decrease steroidogenic functions of isolated adrenocortical cells, as might have been predicted from our earlier results in *S. undulatus* [7]. However, during 10 weeks of food restriction in *S. jarrovi*, growth rate (measured as mm length gain per day) was positive (about 0.1 vs. 0.2 mm/day) in both food-restricted and *ad libitum*-fed (control) lizards of both sexes ( $P < 0.001$ ; [17]), even though body condition was decreased by food restriction [17]. By this measure, lizards in both treatment groups were in positive energy balance, even though food-restricted lizards had negligible fat stores compared to controls [17]. In addition, the laboratory-housed lizards in the present study had unrestricted access to water, in sharp contrast to mid-summer field conditions for *S. undulatus*. Thus, in addition to any inherent species differences, conditions of the present study differed demonstrably from our earlier report on *S. undulatus* [7]. It is perhaps not surprising that the earlier study failed to predict the present experimental responses.

Even though food restriction in the present experiment was sufficient to induce increases in adrenocortical steroidogenic capacities at the cellular level, baseline plasma B did not differ between treatment groups (Fig. 1). In light of the adrenocortical cellular responses, we speculate that baseline adrenocortical stimulation by ACTH in food restricted lizards must have been lower than in controls, although our single point determination of plasma B is obviously not an adequate measure of the dynamic balance between secretion and clearance. By comparison, plasma B was reported to be unaffected by 20 days of food restriction in tree lizards (*Urosaurus ornatus*) [27]. In the same species, 7 days of food restriction actually caused a reported decrease in plasma B [28], whereas an increase would have been predicted on the basis of studies on mammals and birds. In *S. undulatus*, the complete absence of food intake (i.e., zero ration) causes plasma B to increase in hatchlings but not adults within 10 days [26]. Thus, severe food restriction can cause an increase in plasma B in lizards, but this response is apparently difficult to elicit except in hatchlings. The B response to food restriction is highly condition-dependent in lizards and clearly requires further characterization.

Restricted energy/nutrient intake and/or challenges to energy balance can have different effects on adrenocortical function even in closely related species. For example, in contrast to effects in cockerels, dietary protein restriction during growth induces general hypofunction of adrenocortical cell responsiveness to ACTH and angiotensin II in the domestic turkey (*Meleagris gallopavo*) [8]. Furthermore, changes in functional properties of adrenocortical cells in either direction do not necessarily translate to circulating concentrations of B. In protein-restricted cockerels, plasma B was elevated 63–336% above control cockerels depending on strain [13,14,51], consistent with the hyperfunction of cells derived from these birds. However, in protein-restricted turkeys, plasma B was elevated 317% over controls even though there was generalized hypofunction of cells derived from these birds [8]. Indeed, as mentioned above, despite substantial differences in cellular steroidogenic capacities, baseline plasma B concentrations were not different between food-restricted and *ad libitum*-fed *S. jarrovi* (Fig. 1). A more comprehensive assessment of the regulation of circulating corticosteroids should include stress responsiveness, cor-

ticosteroid clearance, turnover, target tissue binding, and corticosteroid binding proteins [4,42,43]. Corticosterone-binding globulin deserves particular attention in this context because of its regulation of the more bioactive free B and the observation that its levels can vary with handling stress [39] and stressors that affect food intake and body condition (e.g., see [2]). Nevertheless, the present study and previous work with dispersed adrenocortical cells make clear that adrenocortical cell function is a highly labile component of this system.

Consistent with previous work on adrenocortical cells from field-active *S. undulatus* [7], adrenocortical cell function differed in some respects between males and females of *S. jarrovi*. Irrespective of feeding regimen, overall P<sub>4</sub> production of female cells was about 46% higher than in males. The significance of this sex difference is at present unclear, but it is not simply an artifact of the *in vitro* system. Overall production of B was equivalent in males and females, and maximal ACTH-induced ALDO production by cells from food-restricted males was about 50% greater than that of food-restricted females (see Section 3.3). In other words, sex differences in adrenocortical cellular steroidogenic functions were hormone- and treatment-specific. Our findings add to a well-established body of work indicating that the lizard adrenal is an important extragonadal source of P<sub>4</sub> [24,25,29,31,33]; indeed, in some lizard species it may be the predominant source of P<sub>4</sub> prior to sexual maturation [33].

According to our iterative polynomial analyses, the response curves of *S. jarrovi* cells are at least biphasic, with the first phase operating at an ACTH EC<sub>50</sub> of  $1.5 \times 10^{-11}$  M, and a second phase operating at an ACTH EC<sub>50</sub> of  $1.3 \times 10^{-10}$  M ( $P < 0.05$ ). Although overall cellular sensitivity to ACTH did not differ between the sexes in response to food restriction, there was a sex difference in the phase contribution to maximal steroid production, specifically B production. The proportional contribution of both phases to maximal steroid production in female cells was essentially unchanged. However, in cells from males, the proportional contribution to maximal ACTH-induced B production shifted to the less sensitive phase ( $P < 0.05$ ). In cells from *ad libitum*-fed males, the proportional contribution of the two phases to B production was roughly evenly split (40% vs. 60%; Table 2). However, in cells from food restricted males, the proportional contribution to B production shifted to the less sensitive phase (17% vs. 83%; Table 2). Thus, even though overall cellular sensitivity (EC<sub>50</sub>) of male cells did not decrease in response to food restriction as measured across the entire dose response curve (see Section 3), the net rate of B production at physiological levels of ACTH ( $\sim 1.5 \times 10^{-11}$  M; 70 mg/ml) could be lower in food restricted than *ad libitum* cells (see Fig. 3). A reduction in B production *in vivo* could be accomplished by shifting the proportion of cellular B response from a phase operating at a near physiological concentrations of ACTH ( $1.5 \times 10^{-11}$  M;  $\sim 70$  pg/ml) to a phase operating at a supra-physiological concentration ( $1.3 \times 10^{-10}$  M;  $\sim 600$  pg/ml). Thus, even though food restriction caused an increase in net maximal rates of production of B, the shift in phase response could explain the lack of effect on plasma concentrations of B.

These two differences in responses of male cells to food restriction, i.e., the effective shift in the maximal B response to the higher EC<sub>50</sub> phase and the 50%-greater maximal ALDO response, may be linked. In these same lizards, Cox et al. [17] reported evidence that the fraction of consumed energy allocated to growth was slightly greater in males than in females, while the fraction allocated to storage was greater in females than in males. Given the roles of B [42,46] and ALDO [44,56] in lipid metabolism and energy balance and partitioning, these findings in lizard adrenocortical cells may reflect sex-specific responses to this particular nutritional stressor to maximize energy allocation for growth.

To our knowledge, the present study and our previous work are the first to suggest “phasing” of steroid responses to ACTH by dispersed adrenocortical cells derived from any species. Thus far, we have observed apparent phasing in *S. jarrovi* and *Sceloporus virgatus* [9] but not in field-active *S. undulatus* [7,9]. It is not clear whether “phasing” is a distributive effect over all adrenocortical cell types, or the result of cellular remodeling of the adrenal gland, producing subpopulations of adrenocortical cells having different sensitivities to ACTH and different ratios of steroid product secreted. Perhaps melanocortin-2-receptor accessory proteins are involved, as they are required for ACTH-receptor deployment and signal transduction and can influence ACTH receptor affinity and cAMP response to ACTH (see [45,50]). Regardless of mechanism, “phasing” may represent another way to dampen adrenocortical cellular responses to ACTH at times when B may be deleterious to survival. Our laboratories are currently investigating these problems. However, the point emphasized by these variations in cellular response to ACTH is that any comprehensive assessment of lizard adrenocortical cell sensitivity to ACTH should include polynomial analysis of dose–response curves. The unique, multi-phasic response to ACTH in lizard adrenocortical cells illustrates the importance of integrating mechanistic studies of stress-induced alterations of HPA components with organismal approaches that address the consequences of adrenocortical stress response on performance and fitness (see [5]).

In summary, we examined the effect of long-term food restriction on adrenocortical cell function in laboratory-housed *S. jarrovi*. Our results show that this prolonged but modest food restriction, while permitting growth, caused an overall increase in adrenal steroidogenic function at the cellular level but did not alter overall adrenocortical cell sensitivity to ACTH. In general, the impact of this nutritional stressor was more apparent with maximal rates than with basal rates of steroid production with a tendency to be greater in male than in female lizards. We conclude that the functional capacities of lizard adrenocortical cells are remarkably labile and must be taken into consideration to explain variations in HPA activity.

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