

Hormonal Response of Male Green Anole Lizards (*Anolis carolinensis*) to GnRH Challenge

JERRY F. HUSAK^{1,2*}, DUNCAN J. IRSCHICK^{2,3}, JUSTIN P. HENNINGSEN³,
KIMBERLY S. KIRKBRIDE¹, SIMON P. LAILVAUX⁴, AND IGNACIO T. MOORE¹

¹Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia

²Department of Biology, University of Massachusetts, Amherst, Massachusetts

³Organismic and Evolutionary Biology Program, University of Massachusetts, Amherst, Massachusetts

⁴Evolution and Ecology Research Centre and School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, New South Wales, Australia

ABSTRACT Circulating plasma levels of testosterone often differ among social classes of sexually mature males within a population, but the general physiological mechanisms underlying such differences remain unclear. Within sexually mature male green anole lizards (*Anolis carolinensis*), smaller “lightweight” males have on average relatively smaller heads, lower bite-forces, and lower testosterone levels compared with larger “heavyweight” males. We conducted gonadotropin-releasing hormone (GnRH) challenges on lightweight and heavyweight males to determine if lightweight males were capable of producing comparable levels of circulating testosterone to heavyweight males but are socially or physiologically suppressed from doing so. We challenged lightweight and heavyweight males with chicken I and II GnRH and measured their resulting levels of testosterone and corticosterone. Neither lightweights nor heavyweights increased circulating testosterone levels after GnRH challenge, suggesting they are already at maximal production levels, consistent with the Challenge Hypothesis. Instead, testosterone levels tended to decrease and corticosterone levels increased, most likely owing to the stress response associated with handling. Our results are dramatically different from GnRH challenges conducted in bird species, suggesting that more field studies are needed in reptilian systems. *J. Exp. Zool.* 311A:105–114, 2009.

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Male secondary sexual characteristics in many vertebrate species are often tightly associated with androgens such as testosterone (Wingfield et al., 2001; Hau, 2007). Such characteristics include morphological and physiological traits, as well as behavior. The influence of testosterone on male aggression is well established, as is the influence of male–male interactions on testosterone secretion (reviewed in Wingfield et al., '90; Oliveira, 2004; Goymann et al., 2007). The Challenge Hypothesis (Wingfield et al., '90) and subsequent extensions of it (reviewed in Hirschenhauser and Oliveira, 2006; Goymann et al., 2007) have gained favor in explaining patterns of male testosterone production in relation to mating system, parental care, and frequency of male–male aggression. Not only

can the Challenge Hypothesis explain variation in testosterone levels among species (Moore et al., 2002; Goymann et al., 2004, 2007), but it can also provide an explanation for disparate patterns of testosterone production in different social classes of sexually mature males within a species (Wingfield et al., '91; Schoech et al., '96; Sinervo et al., 2000). The Challenge Hypothesis predicts that males of seasonally breeding species with

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*Correspondence to: Jerry F. Husak, Department of Biological Sciences, Virginia Tech, 2119 Derring Hall, Blacksburg, VA 24061. E-mail: husak@vt.edu

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polygynous mating systems and a high probability of male–male interaction during the breeding season should maintain maximal levels of circulating plasma testosterone to facilitate the expression of behavioral traits necessary for contest success. As an extension, one would predict that within a species, sexually mature males that are unlikely to successfully compete with rivals in agonistic encounters, and do not engage in such encounters, should not maintain high levels of testosterone, especially if there are detrimental effects associated with elevated circulating testosterone (e.g., Folstad and Karter, '92; Wingfield et al., 2001). If this is the case, researchers should also determine how those males restrict testosterone production. Do lower levels occur via a suppression of the hypothalamic–pituitary–gonadal (HPG) axis or suppression of testis development or seasonal growth (i.e., testosterone production), or both?

Many investigators have used gonadotropin-releasing hormone (GnRH) challenges to discern the maximal responsiveness of the HPG axis (via testosterone production) at a given stage in the reproductive cycle (e.g., Wingfield et al., '79; Moore et al., 2002). GnRH stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate sperm and testosterone production in the testes. GnRH challenges have been successful in elucidating seasonal and individual variation in the maximal capacity to produce testosterone (e.g., Jawor et al., 2006; McGlothlin et al., 2007). Most studies have focused on bird species (Goymann et al., 2007), many of which show a marked increase in testosterone production after injection of GnRH (reviewed in Jawor et al., 2006). Tests of HPG axis responsiveness via GnRH challenge are noticeably lacking in species that are hypothesized to maintain physiologically high levels of testosterone, and, hence, may not increase testosterone levels with GnRH challenge. Males of many temperate iguanian lizard species represent good candidates for such studies, as the majority are territorial, polygynous, and do not display parental care (Stamps, '83). According to the Challenge Hypothesis, such males are expected to maintain high levels of testosterone during the breeding season during which they are likely to interact with numerous sexually receptive females, as well as rival males (Klukowski and Nelson, '98; Smith and John-Alder, '99).

Patterns of GnRH secretion in reptiles are poorly understood, and sensitivities to different

forms of GnRH appear to differ across the major clades of nonavian reptiles. For example, mammalian GnRH injections increase testosterone production in alligators (Lance et al., '85), but neither mammalian GnRH nor chicken-I GnRH increased testosterone production in turtles or snakes (Licht et al., '84). However, single injections of chicken-II GnRH were sufficient to cause significant elevations in plasma testosterone within 20 min in the lizard *Podarcis sicula* that were maintained for over an hour (Ciarcia et al., '89). These disparate results may reflect differences in the underlying GnRH control system, or they may simply reflect which form of GnRH was used in experiments. In either case, it is apparent that turtles and squamate reptiles (snakes and lizards) do not respond to mammalian or chicken-I GnRH in the same manner as mammalian and amphibian laboratory animals (McCreery et al., '82; Licht et al., '84). Because few generalities are available concerning reptilian responses to GnRH, studies such as ours, conducted in a field context, are an important contribution to an understanding of how testosterone production is regulated in reptiles in nature (Goymann et al., 2007).

We investigated the response of free-ranging, sexually mature male green anole lizards (*Anolis carolinensis*) to GnRH challenge. This species has several advantages as a model system for behavioral endocrinology, including an enormous amount of baseline information on neuroendocrine mediators of courtship morphology and behavior (reviewed in Lovern et al., 2004; Wade, 2005) and aggression (Yang and Wilczynski, 2002), as well as substantial information on its behavioral ecology in nature (e.g., Jenssen et al., '95, 2001; Irschick et al., 2005). Another aspect of this species that makes it interesting is the existence of two distinct size classes (presumably reflecting age classes) among sexually mature males within some green anole lizard populations (Lailvaux et al., 2004; Vanhooydonck et al., 2005). These “morphs” differ in relative dewlap size, head shape, and bite-force performance (Vanhooydonck et al., 2005), with smaller “lightweight” males having relatively smaller dewlaps, smaller heads, and lower bite-forces than larger “heavyweight” males (see Lailvaux et al., 2004; Vanhooydonck et al., 2005). These morphological and performance differences appear to be associated with significant differences in circulating plasma testosterone concentrations: lightweights have lower testosterone levels than heavyweights on average (Husak et al., 2007). However, the difference in

testosterone levels between morphs is not discontinuous in the same manner as the morphological differences. Nonetheless, from an endocrine viewpoint these intrasexual differences are intriguing, because they represent a major postmaturation ontogenetic transition in body shape, perhaps owing to changes in hormone profiles at some critical body size at which males can compete for territories. Thus, an important question to ask is whether lightweight males that have low circulating levels of testosterone can produce higher testosterone levels, but simply do not, or whether they are physiologically incapable of producing high levels of testosterone. We addressed this question by challenging lightweight and heavyweight males with GnRH and measuring subsequent testosterone levels.

In this study we conducted GnRH challenges in sexually mature male green anole lizards during the breeding season. We challenged both lightweight and heavyweight males, predicting a difference in testosterone levels in response to GnRH. We hypothesized that heavyweights are maintaining maximal plasma levels of testosterone, as predicted by the Challenge Hypothesis. Thus, if there is social suppression of testosterone production, then we predicted that lightweights would show an increase in testosterone levels, whereas heavyweights would not. However, if lightweights are reproductively suppressed, it is possible that their testes have not matured to the extent that has occurred in heavyweights. That is, lightweight males are able to produce sperm, but they are not producing large amounts of testosterone. Hence, we examined testis size in a cross section of males, ranging from small lightweights to large heavyweights as an index of overall testis maturation. Because the GnRH challenges require substantial handling, we also examined the stress response of males in relation to their HPG axis response to GnRH challenge.

MATERIALS AND METHODS

Study site and field methods

We studied a population of green anoles (Irschick et al., 2005; Bloch and Irschick, 2006) on the Tulane University campus in New Orleans (Orleans Parish). We had two experimental periods, the second being motivated by results from the first. We first conducted GnRH challenges early in the breeding season (May 2007). The second set of GnRH challenges occurred approxi-

mately halfway through the breeding season (June 2007). During both time periods, male lizards were observed to display at females and rival males. We sampled lizards from different areas of the study site during the second set of experiments to avoid recapturing the same individuals from the first set of experiments.

Lizards were captured from clumps of vegetation by hand or noose between 10:00–13:00 hr each day. Within 2 min of capture we collected whole blood from the suborbital sinus with a heparinized microhematocrit capillary tube to assess baseline hormone levels. Blood was collected between 10:00–13:00 hr each day to minimize diel variation in circulating hormone concentrations. If a lizard moved more than 1 m prior to capture, we did not capture that lizard for that day. Once a sample was collected it was transferred to a 0.75-mL microcentrifuge tube and placed on ice until it was returned ≤ 5 hr later to the laboratory, where the plasma fraction (mean volume \pm SEM = 11.1 ± 0.43 μ L) was separated by centrifugation and stored at -20°C until assays were conducted. After initial blood samples were taken, we conducted GnRH challenges, re-sampling individuals later (see below). After GnRH challenges, we measured snout–vent length (SVL) to the nearest 1 mm with a ruler to determine the life-stage morph of each individual. Males with SVL exceeding 64 mm were considered heavyweights, whereas those with SVL less than 64 mm were considered lightweights (following Lailvaux et al., 2004). This SVL value is based on previous statistical analyses of where the “gap” in the bimodal distribution of males occurs (see Vanhooydonck et al., 2005 for details). Lizards were temporarily marked with a dorsal paint spot to prevent recapture within a sampling period. All lizards were returned to their exact point of capture within 2 hr.

GnRH challenges

Lizards were challenged with GnRH to determine their absolute HPG axis responsiveness via changes in plasma levels of testosterone. We used a protocol similar to a previous lizard study that found a testosterone-production response to a single 0.05 μ g dose of GnRH within 20 min (Ciaracia et al., '89). We challenged lizards with the only GnRH form found in the brain of *A. carolinensis* (Lescheid et al., '97), chicken-II LH releasing hormone (cGnRH-II). Challenges were first conducted in May 2007. All GnRH challenges were conducted immediately after the initial blood

sample was obtained (see above). Each lizard was then given an intramuscular injection (Jawor et al., 2006; McGlothlin et al., 2007) of 1.0 μg of cGnRH-II (American Peptide Company 54-8-24) dissolved in 10 μl of phosphate-buffered saline (PBS). We dissolved GnRH in PBS 1 week before experiments were conducted, partitioning the solution into 0.75 microcentrifuge tubes, which were promptly frozen at -20°C until used. Each day of the field trials, we used a fresh microcentrifuge tube of GnRH, kept on ice in a cooler during the duration of the trials. Remaining GnRH was discarded at the end of the trials for a day. The dose used was meant to ensure a response if the HPG axis was capable (Goymann et al., 2007). Each lizard was then placed into an individual plastic bag and held in a larger cloth bag in the shade for 30 min. By keeping lizards in the shade, we were able to maintain lizards at or near their preferred body temperature and therefore prevented overheating. After 30 min, we took a second blood sample. Lizards were then measured and released at their original point of capture. Because studies of other taxa have revealed consistent, dramatic responses to GnRH challenge, we did not include a control group of lizards injected only with PBS (e.g., Jawor et al., 2006; McGlothlin et al., 2007). A control group would have reduced our available experimental sample size and added minimal useful information toward answering the questions of interest. We were not interested in whether there was a difference between lizards responding to GnRH vs. PBS; numerous studies in other taxa have elucidated this difference (e.g., Moore et al., 2002), including lizards (Ciarcia et al., '89). Our design allowed us to examine individual changes in testosterone levels owing to the GnRH challenge, as well as individual changes in corticosterone levels associated with handling stress. Thus, our repeated measures design with preinjection and postinjection blood samples allowed individuals to serve as their own controls. Challenges in May were conducted on the Tulane campus ($n = 10$ lightweights and $n = 10$ heavyweights).

The results from our first set of experiments (see the section "Results" below) lead us to try to rule out our methodology and the form of GnRH as being responsible for the results obtained. Hence, in June 2007, we replicated the experiment on Tulane campus, and this time waited 1 hr before re-sampling individuals that were injected with cGnRH-II ($n = 6$ lightweights and $n = 7$ heavyweights). We also injected a separate group of

lizards ($n = 6$ lightweights and $n = 6$ heavyweights) with 0.5 μg of chicken GnRH-I (Sigma L0637, St. Louis, MO; the active form in passerine birds; e.g., Jawor et al., 2006; Moore et al., 2002) dissolved in 10 μl of PBS after an initial blood sample was taken. One hour after injection we took another blood sample from these individuals, measured them, and released them at their exact point of capture.

Testis size

To determine if there were any differences between the heavyweight and lightweight male morphs in testis size, we measured testes from preserved adult male *A. carolinensis* specimens ($n = 21$) collected during late spring 2003 from a nearby population and drawn from the same individuals used in Lailvaux et al. (2004) (Good Hope Field population; Bloch and Irschick, 2006), in addition to adult males collected at the same site during late spring 2007 ($n = 8$) and preserved with identical procedures as the 2003 lizards. Lizards were removed from collections and towel dried to remove excess alcohol. We then weighed each specimen to the nearest 0.01 g using a Denver instruments M-220 electronic balance (Denver, CO), and measured SVL to the nearest 0.01 mm using Mitutoyo electronic calipers. We then dissected the specimens and removed their left and right testes, which were weighed to the nearest 0.001 g. We first used general least-squares regression to determine whether body size (SVL) was a significant predictor of average testis size. To test whether larger males have relatively larger testes, we regressed both average testis mass and body mass against SVL, and then tested whether residual body mass significantly predicted residual testis mass. We used independent-sample *t*-tests to compare absolute and residual testis mass between lightweights and heavyweights.

Hormone assays

Concentrations of testosterone and corticosterone were measured by standard radioimmunoassay (RIA) techniques following extraction and chromatographic separation (Wingfield and Farnner, '75; Moore et al., 2000b). May samples were run in one assay, and June samples were run in a second assay. However, our interest was not in comparing hormone levels between sampling periods, but instead to examine changes in testosterone and corticosterone within a sampling period. Greater detail of our RIA techniques are

described elsewhere (Husak et al., 2007). For individual extraction efficiency determination, we equilibrated each sample overnight with 2,000 cpm of tritiated steroid. Each sample was extracted with 5 mL of distilled dichloromethane and resuspended in 10% ethyl acetate in isooctane. Chromatographic separation of steroids was accomplished with a series of solutions passed through columns with increasing concentrations of ethyl acetate in isooctane. Testosterone and corticosterone fractions were collected, and the rest were discarded. After this, samples were dried in a 40°C water bath under nitrogen gas, resuspended in 600 µL phosphate buffered saline, and maintained overnight at 4°C. Individual extraction efficiency for each steroid (mean recoveries were 61 and 60% for testosterone and 73 and 68% for corticosterone) was determined from 100 µL of the sample while 200 µL of the sample was allocated to each of two duplicates for the assay. Serial dilutions for the standard curves were performed in triplicate (range of curves: testosterone: 500–1 pg; corticosterone: 2,000–4 pg). All samples were then incubated overnight with 100 µL of antiserum (testosterone: WLI-T-3003S, Fitzgerald Industries, Concord, MA; corticosterone: Esoterix Endocrinology, Calabasas Hills, CA 91301) and 100 µL of tritiated steroid (approximately 10,000 cpm). Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual recovery. Intra-assay coefficients of variation (CV) for testosterone were 6 and 13% for May and June samples, respectively. Intra-assay CVs for corticosterone were 18 and 22% for May and June samples, respectively. Inter-assay CVs were 7% for testosterone and 13% for corticosterone.

Statistical analysis

We used a series of paired-sample *t*-tests to examine differences in circulating plasma testosterone and corticosterone (analyzed separately) before and after injection with GnRH. Each of the four experiments was examined separately, as we did not wish to statistically compare different experiments. Within each of the experiments, lightweights and heavyweights were analyzed separately. Hormone data were log₁₀-transformed to meet assumptions of normality. We used Pearson correlation analysis to determine if preinjection testosterone levels were correlated

to postinjection level. We also used Pearson correlation analysis to determine if the change in testosterone levels (preinjection minus postinjection levels) was related to change in corticosterone levels (postinjection minus preinjection levels). We note that the change in testosterone levels, as calculated, represents the decrease in testosterone levels, whereas the change in corticosterone levels represents the increase in corticosterone levels.

RESULTS

Morph differences in response to GnRH challenges?

Testosterone concentrations 30 min following injection with cGnRH-II did not significantly differ from preinjection concentrations in May (Table 1) for either lightweights ($t = 1.96$, $df = 9$, $P = 0.08$) or heavyweights ($t = 1.16$, $df = 9$, $P = 0.28$). Indeed, there was a trend for testosterone levels to be lower after the GnRH injection, presumably associated with the stress of capture and handling (e.g., Moore et al., 2000a). In June, 1 hr after cGnRH-II challenges, both lightweights ($t = 2.77$, $df = 5$, $P = 0.039$) and heavyweights ($t = 3.38$, $df = 6$, $P = 0.019$) had significantly reduced testosterone levels (Table 1). One hour after cGnRH-I (Table 1), heavyweights ($t = 0.64$, $df = 5$, $P = 0.55$) did not differ in testosterone levels, but lightweights approached a significant reduction ($t = 2.47$, $df = 5$, $P = 0.057$). Mean difference in testosterone levels (pre–post) did not differ between lightweights and heavyweights in any of the experiments ($P > 0.21$ for all). We note that average values of testosterone were similar between lightweights and heavyweights in the May sample, but the trend (two-sample *t*-test, $P = 0.11$) was for heavyweights to have higher testosterone, consistent with our previous findings (Husak et al., 2007). The similarity is likely owing to small sample sizes, as the differences previously observed were owing to subtle differences in body size between the groups. In any case, this does not change the validity of our findings that GnRH did not increase testosterone production in either group, in contrast to what has been found in other taxa, including a study on lizards with similar sample sizes for each experimental group ($N = 10–15$) (Ciarcia et al., '89).

In all cGnRH-II challenge experiments, with lightweights and heavyweights pooled owing to a lack of other differences, preinjection and postinjection testosterone levels were significantly and positively correlated ($r > 0.70$, $P < 0.001$ for both).

TABLE 1. Response to GnRH challenge in lightweight and heavyweight male green anoles during the breeding season in southeastern Louisiana

	Lightweights		Heavyweights	
	Preinjection	Postinjection	Preinjection	Postinjection
May cGnRH-II (30 min)				
Testosterone	8.67 ± 2.59	5.89 ± 1.12	10.90 ± 2.24	7.81 ± 1.40
Corticosterone	8.05 ± 1.21*	32.23 ± 8.64*	7.47 ± 0.61*	20.08 ± 3.95*
June cGnRH-II (1 hr)				
Testosterone	6.15 ± 2.84*	1.38 ± 0.49*	12.41 ± 6.80*	3.04 ± 1.60*
Corticosterone	3.18 ± 0.61*	23.55 ± 5.50*	2.11 ± 0.51*	26.01 ± 6.11*
June cGnRH-I (1 hr)				
Testosterone	2.38 ± 0.79	0.79 ± 0.11	3.62 ± 1.73	3.23 ± 1.62
Corticosterone	3.29 ± 0.61*	17.46 ± 4.86*	4.44 ± 2.20*	27.48 ± 7.96*

Numbers represent mean circulating plasma testosterone and corticosterone concentrations (ng/mL ± 1 standard error of the mean) before (pre) and after (post) injection with GnRH. Asterisks denote a significant difference between preinjection and postinjection values.

However, in the June cGnRH-I challenges, initial and final testosterone levels were not correlated, though the relationship approached significance ($r = 0.55$, $P = 0.066$).

Morph differences in stress response?

Corticosterone concentrations were significantly higher 30 min following injection with cGnRH-II in May (Table 1) for both lightweights ($t = 4.55$, $df = 9$, $P = 0.001$) and heavyweights ($t = 3.41$, $df = 9$, $P = 0.008$). In June, 1 hr after cGnRH-II challenges, both lightweights ($t = 3.67$, $df = 5$, $P = 0.014$) and heavyweights ($t = 4.37$, $df = 6$, $P = 0.005$) had significantly higher corticosterone levels (Table 1). Similarly, 1 hr after cGnRH-I both lightweights ($t = 3.34$, $df = 5$, $P = 0.021$) and heavyweights ($t = 3.60$, $df = 5$, $P = 0.016$) had significantly higher corticosterone levels (Table 1). Mean difference in corticosterone levels (post minus pre) did not differ between lightweights and heavyweights in any of the experiments ($P > 0.23$ for all).

In the May cGnRH-II challenge experiment, and in the June cGnRH-I challenge experiment, with lightweights and heavyweights pooled owing to a lack of other differences, preinjection and postinjection corticosterone levels did not significantly correlate ($r < 0.34$, $P > 0.13$ for both). However, in the June cGnRH-II challenges, initial and final corticosterone levels were significantly and positively correlated ($r = 0.72$, $P = 0.005$).

Response of testosterone to increased corticosterone

We pooled all experiments to determine whether final (i.e., after injections) testosterone concentra-

tions were correlated with final corticosterone concentrations, but we detected no significant relationship ($r = 0.09$, $P = 0.56$). Similarly, we pooled all experiments to test whether the difference in testosterone concentrations (prelevel minus postlevel; the magnitude of decrease) was correlated to the difference in corticosterone concentrations (postlevel minus prelevel; the magnitude of increase). We found no significant relationship between the two ($r = -0.20$, $P = 0.17$). We also found no significant relationship when percent changes in testosterone and corticosterone were examined ($P = 0.8$).

Testis size

SVL ($r^2 = 0.58$, $F_{1,27} = 37.92$, $P < 0.001$; Fig. 1) was a significant predictor of average testis mass. However, residual body mass was not a significant predictor of residual testis mass ($r^2 = 0.04$, $F_{1,27} = 1.04$, $P = 0.32$). Heavyweights had significantly larger testes on average than lightweights ($t = 4.79$, $df = 27$, $P < 0.001$), but analysis of residual testis mass revealed no significant differences ($t = 1.24$, $df = 27$, $P = 0.23$). Nonetheless, these results show that overall larger, and hence older, lizards have heavier testes than smaller, younger lizards.

DISCUSSION

Our results revealed several key findings. First, male green anoles did not increase circulating testosterone levels after GnRH challenge. This result was consistent when challenging the males with two forms of GnRH (chicken-I and chicken II), challenging males during two time periods in the breeding season, and varying the amount of

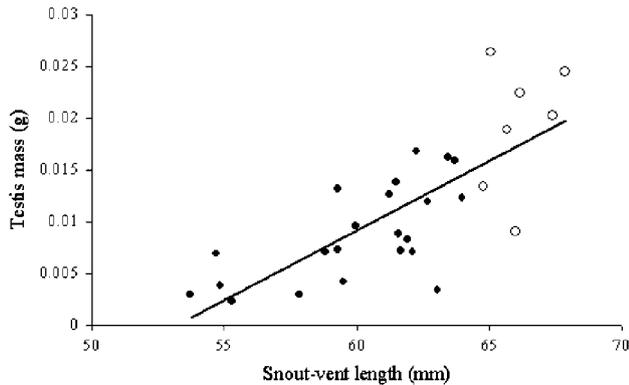


Fig. 1. Relationship between snout-vent length and average testis mass in adult male green anole lizards (*Anolis carolinensis*). Lightweights are represented by filled circles, and heavyweights by open circles. Regression equation: $y = 0.0013x - 0.0717$; $r^2 = 0.58$, $P < 0.001$.

time for the HPG axis to respond (30 and 60 min). Second, there was no difference between lightweights and heavyweights in the testosterone response to GnRH challenge. Neither morph increased testosterone, rather both tended to decrease circulating testosterone levels. Third, as expected, corticosterone concentrations more than quadrupled over the duration of the experiments, presumably owing to handling stress. However, the magnitude of the stress response was not a consistent predictor of the decrease in testosterone concentrations, nor did it differ between morphs. Finally, testis size was significantly predicted by body size, with lightweights having significantly smaller testes than heavyweights.

The Challenge Hypothesis (Wingfield et al., '90) predicts that males of polygynous, territorial species without parental care should maintain high plasma levels of circulating testosterone throughout the breeding season without a peak associated with social interactions. Our results are consistent with this hypothesis. Previous tests with territorial intruders in polygynous lizard taxa have also supported the Challenge Hypothesis. In general, male lizards challenged with an "intruder" rival did not respond with an increase in testosterone, suggesting that male lizards maintain consistently high levels of circulating testosterone for such situations (Moore, '87; Thompson and Moore, '92; Knapp and Moore, '95, '96; Klukowski and Nelson, '98; Woodley et al., 2000; see also Schuett et al., '96). However, Smith and John-Alder ('99) found elevated testosterone levels in male *Sceloporus undulatus* after intrusions. As these authors point out, the use of enclosures, as opposed to free-ranging individuals, during the

experiments make comparison to other studies difficult. Another exception to the finding that male lizards do not increase testosterone after social challenge was in a study of laboratory-housed male green anoles (Greenberg and Crews, '90), where increased plasma testosterone levels were detected within 1 hr following intrusions. It is possible that the laboratory social environment, with a lack of frequent intraspecific encounters, caused initially low baseline testosterone levels, and the sudden intrusions caused an elevation approaching what would occur in free-ranging animals. In accordance with this hypothesis, the baseline (i.e., control) testosterone levels that Greenberg and Crews ('90) found were much lower than those found in free-ranging anoles (Jenssen et al., 2001; Husak et al., 2007), and the average level of elevated androgens after intrusion was only about half that seen in field-active lizards. Despite these methodological differences, the study by Greenberg and Crews ('90) is valuable, because it suggests that high levels of testosterone, typical of lizards in the field, are due in large part to the social environment and the likelihood that they will frequently interact with females and rival males (Wingfield et al., '91; Goymann et al., 2007).

We found no elevation in plasma testosterone after GnRH challenge, but previous work has revealed that it is possible to stimulate testosterone production in green anoles. Licht and Tsui ('75) caused a significant increase in plasma testosterone after injection of laboratory-housed males with mammalian FSH. The testosterone values they recorded (>150 ng/mL on average) were more than triple that reported in free-ranging males from our population (Table 1; see also Husak et al., 2007) and in another well-studied population (Jenssen et al., 2001). There are no available data to explain this discrepancy, but we suggest several testable hypotheses. First, our dose of GnRH may have been insufficiently high to increase testosterone production. However, this is unlikely as lower doses have caused an increased HPG axis response in other lizard species (Ciarica et al., '89; Shanbhag et al., 2000). It is also unlikely that our high dose was inhibitory, as a dose-response study in mammals showed plasma testosterone to increase with a range of doses spanning an order of magnitude (Roser and Hughes, '92). Dose responses to GnRH in a laboratory setting are not well studied in squamate reptiles. The differences in mammalian and squamate HPG axes may make comparisons difficult,

though. For example, rats and humans are known to have GnRH receptors in the testes (Petersson et al., '89; Kakar et al., '94), but the presence of such a receptor in squamate reptiles is relatively unknown. Second, there may be a threshold number of GnRH receptors in the pituitary that prevented further release of FSH and LH in our population. This could be owing to seasonal or population differences in brain physiology (e.g., Canoine et al., 2007). Alternatively, the same could be true of FSH or LH receptors in the testes. A third hypothesis is that the stress response provided an "override" of any potential increase in testosterone (Moore et al., 2002). Although increases in corticosterone owing to stress have been causally linked to concomitant decreases in testosterone in lizards (Knapp and Moore, '97), this relationship is not universal across taxa (Moore and Jessop, 2003; Goymann and Wingfield, 2004), or even within different social groups within taxa (Knapp and Moore, '97; Husak et al., 2007). We note that our results should be interpreted with caution because the HPG axis was being manipulated with elevated levels of GnRH. Although we did not find a relationship between corticosterone increases and testosterone decreases, this relationship could have been obviated by individual variation in HPG susceptibility to increased adrenal activity. Future experimental studies will help clarify these issues. Nonetheless, we note that this represents the first study to our knowledge that attempted GnRH challenges in a free-ranging population of squamate reptiles in a manner comparable to the standard protocols used in bird studies (e.g., Goymann et al., 2007). Our results are noteworthy because they are very different from the "norm" found in bird GnRH challenges.

Contrary to our prediction, lightweight males, like heavyweights, did not increase testosterone levels after GnRH challenge, regardless of experimental conditions. Therefore, the finding that lightweights have lower testosterone on average than heavyweights (Husak et al., 2007) may be owing to an inability of some lightweights to produce the high levels documented in heavyweights. We propose several explanations. First, lightweights may have fewer receptors to GnRH in the pituitary or FSH/LH in the testes compared with heavyweight males. However, this scenario was not supported by other data in reproductively suppressed mole-rats (Bennett et al., 2000). However, subfertile stallions did not increase plasma testosterone concentrations after GnRH challenge

despite an increase in LH and FSH (Roser and Hughes, '92). As fertile stallions did show an increase in plasma testosterone after GnRH challenge, these results suggest that the HPG axis response can differ among classes of males at the level of the testis response. A second hypothesis is that testis maturation of lightweights is delayed compared with heavyweights, thus precluding the production of equivalent testosterone levels. Our finding that smaller males have smaller testes provides some supporting evidence for this hypothesis, though data linking testis size with testosterone production are needed. We found that larger males had larger testes, but not disproportionately so. Thus, body size alone appears to account for this difference, making it somewhat difficult to suggest a suppressive effect specifically. Complicating this hypothesis is the fact that testes are primarily composed of Sertoli cells for sperm production, with far fewer Leydig cells for testosterone production. However, even though Leydig cells do not comprise the majority of testis mass, the differences in testis size between lightweights and heavyweights (Fig. 1) underscore potential differences in maturation and testosterone production. The causal relationships among testosterone levels, testis size, and social behavior remain unclear, but we suggest that smaller males are typically not capable of competing with larger males (Lailvaux et al., 2004); thus, smaller males may not invest in testis enlargement and the associated detrimental effects of increased plasma testosterone levels (e.g., Folstad and Karter, '92; Wingfield et al., 2001) until they are larger in size and can compete for access to females. This is consistent with previous work in the green anole (Licht and Pearson, '69; Pearson et al., '76). The susceptibility of male reptilian reproductive physiology to environmental factors is well known (reviewed in Duvall et al., '82), and studies in other taxa have shown that the social environment can feedback to influence reproductive physiology (e.g., cooperative breeders, Fitzpatrick et al., 2006). In species that show life-stage "morphs" with different reproductive strategies (Irschick and Lailvaux, 2006), social suppression may reach beyond behavior to underlying physiological and reproductive potential. Among lizards, there are documented examples of such age-related patterns of social suppression of territoriality and access to females (Pratt et al., '94; Baird and Timanus, '98; Lailvaux et al., 2004). More work is greatly needed to determine how the social environment interacts

with testis development, testosterone production, and subsequent social behavior of socially suppressed males.

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