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Social Modulation of Androgens and Glucocorticoids in Territorial Male Collared Lizards

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Social Modulation of Androgens and Glucocorticoids in

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TABLE OF CONTENTS

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Figure Page

Table Page

Abstract

The challenge hypothesis predicts that breeding strategy and degree of parental care may modulate within season hormone levels and androgen responsiveness to social challenges in males. Hirschenhauser and Oliveira (2006) expanded this theoretical framework to separately test the roles of sexual, paternal, and agonistic behaviors on androgen responsiveness. The social stress hypothesis predicts that males exposed to repeated challenges will increase corticosterone levels which may have an antagonistic effect on testosterone level. To test these hypotheses, I measured plasma testosterone and corticosterone in male collared lizards over the activity season (April-July) to examine potential variation in Baseline hormone levels. I also exposed resident territorial males to either 15 min conspecific intrusions or an empty noose-pole over four consecutive days to examine androgen and glucocorticoid responsiveness to Acute (Day 1) and Chronic (Days 2, 3, and 4) challenges. Inconsistent with the predictions of the challenge hypothesis, males did not maintain peak testosterone levels across the activity season. Testosterone levels peaked in April and June, and were significantly lower in May and July. This temporal pattern of testosterone concentrations

is similar to the temporal pattern of courtship activity reported by a previous study on males in the same population. Males that were challenged by an intruder responded more aggressively than controls in June, but not in May, perhaps because during June males are courting females frequently. Males did not increase testosterone in response to challenges, and even exhibited a tendency for decreased testosterone between baseline and acute experimental phases. These results are consistent with Hirschenhauser and Oliveira's (2006) finding that agonistic behavior does not consistently prompt increased androgen levels among vertebrates. Decreased testosterone in collared lizards may be attributed to the threat of losing social dominance, or the reduction in courtship activities during challenges. Temporal correspondence of high testosterone levels in my study with periods of peak courtship frequency reported by Baird et al. (2001), suggests that courtship may prompt secretion of androgens. Corticosterone levels did not increase following single or repeated intrusions, which does not support the predictions of the social stress hypothesis, or allow for the possibility of an antagonistic interaction between corticosterone and testosterone in collared lizards. Two possibilities for the lack of a stress response to

challenges are that intruder males were not perceived as a threat, or that I may not have measured the most biologically relevant form of corticosterone.

Introduction

The endocrine system is a powerful modulator of reproductive physiology, morphology, and behavior in male vertebrates (Adkins-Regan 2005; Balthazart 1983; Wingfield and Ramenofsky 1985). Most research has focused on androgens (primarily testosterone) and glucocorticoids (primarily corticosterone). Because androgens and glucocorticoids have different functions (Adkins-Reagan 2005), there is separate literature on the relationship between behavior and each class of hormone. Below I review the pertinent literature for each, first for androgens and then for glucocorticoids.

Androgen Modulation of Social Behavior

The major hypothesis proposed to explain the role of androgens in mediating physical and behavioral differentiation of the sexes is the Organizational - Activational Model (Arnold and Breedlove 1985; Moore 1991; Phoenix et al. 1959). This model proposes that androgens permanently organize neural pathways within the central and peripheral nervous systems during a critical period early in life. In male vertebrates, organizational effects of androgens stimulate development and growth of skeletal muscle (Ketterson et al. 1992) and induce spermatogenesis

(e.g. Brown and Follett 1977). Later, typically during adulthood, short-term changes in steroid hormones “activate” neural pathways in the brain (often that were organized earlier), promoting short-term activation of sex-specific behavior patterns such as courtship and aggression (Adkins-Regan 2005; Arnold and Breedlove 1985; Moore 1991; Phoenix et al. 1959).

Testosterone is the primary hormone that mediates breeding-season aggression and reproductive behavior in males (Adkins-Regan 2005). Testosterone stimulates display, patrol (Chandler et al. 1994; Schoech et al. 1999), and vocalization while mediating call duration (Nottebohm et al. 1987), and the frequency and intensity of courtship and aggression (Arnold 1975; Balthazart 1983; Wingfield and Moore 1987; Wingfield et al. 1990. Testosterone implantation enhances these behavioral characteristics in some species, whereas castration in others reduces them as a result of decreased testosterone production (e.g. Balthazart 1983; Harding 1983; Harding et al. 1988; Moore 1988).

Social Modulation of Androgens

Although it is clear that testosterone may have strong activational effects on male behavior (Balthazart 1983;

Fusani et al. 2007; Hau et al. 2000; Wingfield and Ramenofsky 1985), interactions between conspecifics, such as male-male contests or courtship of receptive mates can feed back to prompt the secretion of additional testosterone (Goymann et al. 2007; Harding 1981; Hirschenhauser et al. 2003; Hirschenhauser and Oliveira 2006; Ketterson et al. 1992; Wingfield et al. 1987; Wingfield et al. 1990.

Although the effects of social stimuli on hormone levels have been tested in most vertebrate taxa, avian studies comprise the majority of research in this area. As an early example, Wingfield (1984b) observed different patterns of testosterone in captive versus free-living male song sparrows. Captive males isolated from social stimuli and exposed to a photoperiod typical of the breeding season maintained stable baseline testosterone levels over time. By contrast, free-living males exposed to receptive mates and male competitors exhibited testosterone concentrations above the baseline level observed in captive males, and varied significantly over the breeding season (Wingfield 1984a). Based upon these observations, Wingfield et al. (1990) proposed the challenge hypothesis (Table 1) to explain how social factors influence testosterone levels over the course of the breeding season.

Hypothesis	Predictions	
	Monogamous Males	**Polygynous Males**
Challenge Hypothesis (Wingfield et al. 1990)	Provide some degree of parental care of offspring.	Provide limited or no parental care of offspring.
	Testosterone is elevated during territory establishment and courtship; testosterone decreases when males are caring for offspring.	Testosterone is elevated early in the activity season and is maintained at physiological maximum levels.
	Males increase testosterone in response to social challenges.	Males do not increase testosterone in response to social challenges.
	Monogamous and Polygynous Males	
Social Stress Hypothesis (Soto-Gamboa et al. 2005)	Acute exposure to a stressor will initiate an increase in corticosterone.	
	Chronic exposure to a stressor will initiate an increase in corticosterone and a decrease in testosterone.	

Table 1. Predictions for seasonal and challenged levels of testosterone and corticosterone for polygynous and monogamous males.

Challenge Hypothesis

Wingfield et al. (1990) proposed that different breeding strategies and degrees of paternal care mediate species differences in androgen responsiveness to social cues (Table 1). When the breeding season begins, male androgen levels are expected to rise from a non-breeding Level A to an elevated breeding season Level B (see Figure 1). Androgens at Level B are hypothesized to support normal reproductive physiology, seasonal development of secondary sexual characteristics, and the expression of some reproductive behavior. However, this level is not expected to be sufficient to stimulate male-male aggression or courtship (Goymann et al. 2007; Goymann 2009; Wingfield et al. 1990).

In species where males provide substantial parental care, androgen levels are expected to increase above Level B toward the physiological maximum (Level C) only during periods of social instability such as territory establishment, conspecific challenges, or courtship (Goymann et al. 2007; Goymann 2009; Wingfield et al. 1990; Figure 1). By contrast, the challenge hypothesis predicts that polygynous territorial males that provide little or no parental care should maintain testosterone levels close to the physiological maximum (Level C) throughout the breeding

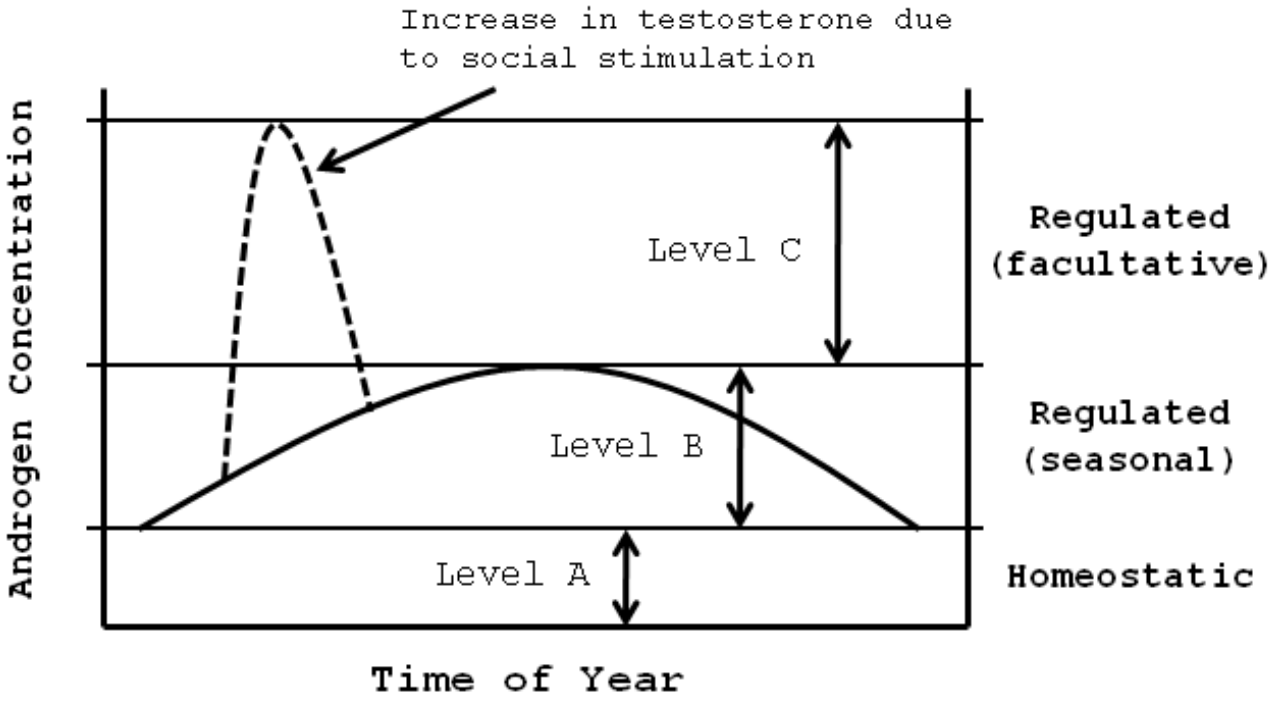

Figure 1. Hypothetical model of changes in androgen concentration as a function of time of year following Wingfield et al. (1990). Level A depicts the homeostatic baseline, Level A to B represents the range of androgen levels required for reproduction, and Level B to C illustrates the range of androgen increase by males following social stimulation. Changes in androgens from Level A to B are proposed to be primarily due to environmental cues (e.g. photoperiod), whereas increases in androgens from Level B to Level C are considered to strictly result from male-male conflict or courtship. Figure redrawn from Goymann and Wingfield 2004.

season to support increased aggression and courtship behavior (Wingfield et al. 1990). As a result, androgen levels should not increase during social instability because testosterone is already at its maximum.

Since the introduction of the challenge hypothesis, several questions have been raised from the results of hundreds of studies across all vertebrate taxa. The challenge hypothesis combines the modulatory roles of male-male aggression, courtship, and paternal investment on androgen responsiveness, however, studies that examine each behavioral context separately may provide a better explanation for their specific influences on androgens. Hirschenhauser and Oliveira (2006) expanded the theoretical framework of the challenge hypothesis to test the roles of sexual, paternal, and agonistic behaviors separately on androgen responsiveness from 196 studies, including 168 vertebrate species. To test the generality of the predictions of the challenge hypothesis, Hirschenhauser and Oliveira (2006) conducted a meta-analysis, calculating the effect size for each of the above social variables on androgen responsiveness. Effect sizes are calculated as the difference between the means of two groups (experimental versus control), divided by their pooled standard

deviation. The magnitude of effect sizes are interpreted as small, moderate, large, and very large.

Hirschenhauser and Oliveira (2006) examined androgen responsiveness to agonistic interactions in two contexts: 1) dominant-subordinate social status, and 2) high group density, both of which are assumed to be a measure of situational male-male aggression. The results of their study revealed that the effect size for agonistic context was only moderate and inconsistent with the predictions of the challenge hypothesis. Several likely reasons include potential confounds with high variability in the contexts in which aggression occurs in the diverse sample of species included in the analysis, and by combining studies that used effects of social status with those using density (Hirschenhauser and Oliveira 2006). Analysis of studies that consider dominance-subordinance status alone is probably a better measure of situational male-male aggression, and thus a better indicator of agonistic context. Although androgen levels increased in response to aggression in some species (Cardwell and Liley 1991; Creel et al. 1993; Greenberg and Crews 1990), the overall effects of male aggression on testosterone failed to support the predictions of the challenge hypothesis when investigated using meta-analysis (Hirschenhauser and Oliveira 2006).

Hirschenhauser and Oliveira (2006) also examined the effect of sexual behavior on androgen responsiveness. Their meta-analysis revealed that high testosterone levels corresponded strongly with the expression of high rates of sexual behavior, particularly in species where males do not provide parental care. Whether males were polygynous or monogamous played less of a role. Evidence that courtship mediates temporal and individual variation in testosterone (Sakata et al. 2003) includes increased levels when mates become receptive (Wingfield et al. 1990), differences depending on the number of broods produced (Wingfield et al. 1990), and individual mating interactions (Kempenaers et al. 2008, but see Moore and Kranz 1983). Courtship encounters also increased the production and release of gonadotropin releasing hormone which stimulates testosterone secretion (Adkins-Regan 2005). Collectively, these studies strongly suggest a role of courtship as a mediator of androgen production which is consistent with the influence of sexual context revealed by Hirschenhauser and Oliveira's (2006) meta-analysis.

The majority of studies testing the challenge hypothesis have only compared seasonal changes in testosterone level to seasonal changes in male behavior patterns, and relatively few studies have experimentally

examined changes in male testosterone and behavior following staged territorial intrusions. Even fewer have measured changes in testosterone and behavior in the same individual under different social conditions (Goymann et al. 2007). Therefore, additional studies are necessary to add to the available data base, particularly in the reptilia, which represented only three of the 168 species studied in Hirschenhauser and Oliveira's (2006) meta-analysis.

Squamates and the Challenge Hypothesis

Among squamate reptiles, the challenge hypothesis has been tested primarily in Iguanian lizards, with two exceptions being copperhead snakes (Viperidae) (Schuett et al. 1996), and the jacky dragon (Agamidae) (Watt et al. 2003). In most lizards, males are polygynous through defense of territories and parental care is rare. Testosterone concentrations typically rise early in the breeding season and are then maintained close to the physiological maximum (Level C; Figure 1) to support territory defense and courtship of females (Hews 1990; 1993; Moore 1988; Moore and Lindzey 1992; but see Thompson and Moore 1992). Because testosterone levels are elevated for extended periods, Thompson and Moore (1992) proposed

that polygynous squamates may be hormonally insensitive to conspecific challenges (Table 1).

Consistent with Thompson and Moore's (1992) extension of the challenge hypothesis (insensitivity hypothesis; Table 1), male testosterone levels did not increase despite behavioral responses to challenges in Yarrow's spiny lizard (Moore 1987; 1988), ornate tree lizards (Knapp and Moore 1995; Thompson and Moore 1992), northern fence lizards (Klukowski and Nelson 1998), and free-living green anoles (Husak et al. 2009). By contrast, captive male green anoles (Greenberg and Crews 1990) and fence lizards (Smith and John-Alder 1999) increased androgen levels following social instability. These mixed results suggest that maintenance of maximal testosterone levels throughout the activity season may not be the rule in polygynous lizards, perhaps because maintaining a chronic high level of testosterone to support territoriality, aggression, and courtship is costly. These behavior patterns are energetically expensive and increase the risks of predation and injury, as well as take time away from other activities such as self maintenance (Dufty 1989; Marler and Moore 1988). Chronically high levels of testosterone may also suppress immune function (Dhabhar and McEwen 1997). Consequently, some lizards may manage these costs by maintaining

testosterone at lower levels, and increasing aggression and testosterone only when necessary.

Predictions of the Challenge Hypothesis for Collared Lizards

Because male collared lizards are territorial and do not provide parental care, the challenge hypothesis predicts that androgen levels across the activity season (early April to June) will be elevated close to physiological maximum to support territorial defense and courtship. Therefore, androgen levels are not expected to increase when territorial males are challenged (Wingfield et al. 1990), even when intraspecific encounters are known to increase the intensity and frequency of aggression in this species (Fox and Baird 1992; Husak and Fox 2003; Schwartz et al. 2007). Rates of advertisement display and courtship activities do, however, vary throughout the activity season in males at Arcadia Lake (Baird et al. 2001; 2003). Male courtship activity peaks during April when 2 year and older females are producing their first clutches, and again in June when 2 year and older females are producing their second clutches and first-year females are producing their first (Baird et al. 2003). Males at this site acquire territories at the beginning of the

activity season without frequent overt aggression (proximal encounters with rivals), and exhibit a distinct peak in advertisement display and patrol in June (Baird et al. 2001; 2003). Consequently, if testosterone is positively associated with territorial defense or courtship, then levels of testosterone should also vary over time. If testosterone levels do vary temporally, then during periods when testosterone levels are lower, males may be able to respond to staged social challenges with increases in testosterone.

A previous study on hormone levels in male collared lizards revealed that differences in baseline testosterone were not correlated with differences in frequencies of baseline behavior patterns (display, courtship, aggression; Baird and Hews 2007). Furthermore, testosterone did not increase in a sample of males exposed to both low and high aggression conditions (male removals; Baird and Hews 2007), which lends support to the prediction that male collared lizards may not increase testosterone in response to challenges. However, the Baird and Hews (2007) study was not designed to measure short-term androgen responses to social challenges. Testosterone levels were measured only once during the midst of the breeding season (late April to June), when females were producing eggs and males were

courting and mating with females, whereas behavior was sampled periodically throughout this time. Sampling hormones only once is likely not a good measure of possible short term hormone responses to social challenges (Baird and Hews 2007).

Finally, maintaining elevated testosterone levels over the activity season is potentially costly. In species that live for multiple seasons (e.g. marine iguanas, Wikelski et al. 2005)these potential costs may lead to reduced life-time fitness (Wingfield et al. 2001). In males of some species, exogenous testosterone increases aggressive behavior, conspicuousness, energetic demands, risk of injury, parasitism, and reduces immune function, body mass, and survival (Hillgarth et al. 1997; Marler and Moore 1988). Because male collared lizards live an average of 3.5 years (Baird et al. 2003), decreased testosterone levels during portions of the activity season may reduce the cumulative costs of maintaining high testosterone levels.

To test the extent to which male polygynous lizards lacking paternal care respond or do not respond hormonally to a social challenge, it is necessary to investigate the relationship between hormone levels and behavior in additional species. I tested two predictions of the challenge hypothesis in territorial male collared lizards.

I measured baseline testosterone levels each month throughout the activity season to test whether androgen levels remained constant as predicted by this hypothesis. I also used experimental introductions of tethered male intruders to test whether or not increased aggression prompted short-term hormonal responses in male territory owners.

Glucocorticoids and the Social Stress Hypothesis

Stress has been defined as a “threat, real or implied, to the psychological or physiological integrity of an individual” (McEwen 2000). The stress response involves: 1) the environmental stimuli that cause stress (stressors), 2) the physiological and psychological changes caused by those stimuli (stress response), and 3) chronic effects that occur as a result of long term exposure to stressors (reviewed in Romero and Butler 2007). Stress may be prompted by behavioral and physiological demands induced by predictable events such as transitions between breeding versus non-breeding periods, migration versus non-migration periods, or shifts in photoperiods (Wingfield et al. 1998). Stress can also be prompted by unpredictable events including food shortages, inclement weather, or agonistic interactions with conspecifics (Greenberg and Wingfield

1987; Greenberg 2003; Goymann and Wingfield 2004; Landys et al. 2006; McEwen and Wingfield 2003; Romero et al. 2009; Sapolsky 1992; Wingfield et al. 1998).

Because glucocorticoids play a large role in maintaining homeostasis (Goymann and Wingfield 2004; Landys et al. 2006; McEwen and Wingfield 2003), perhaps it is not surprising that they also may mediate metabolic changes during predictable and unpredictable life history transitions (Goymann and Wingfield 2004; Landys et al. 2006; McEwen and Wingfield 2003; Romero et al. 2009). Corticosterone in particular, has been implicated in the induction of feeding prior to migration, and the mobilization of glucose necessary to support increased locomotion and breeding (Landys et al. 2006). Facultative responses to unpredictable, emergency events (e.g. disease, human disturbance, social interactions) involve increasing glucocorticoid production above that observed during predictable events (McEwen and Wingfield 2003). Persistence of stressors and failure to habituate often results in chronically elevated glucocorticoid levels (Goymann and Wingfield 2004; McEwen 2000).

The ability to mediate metabolic systems at more than one stable state through endocrine, neural, and immune responses is known as allostasis (Landys et al. 2006;

Sterling and Eyer 1988). Allostatic load, therefore, is the cumulative demand to respond to a suite of stressors. High allostatic load may result either when levels of stress hormone are high chronically, or the stress system does not operate efficiently in response to adverse psychosocial or physical situations. Allocation of energy to immediate activities without replenishment of stores increases allostatic load (Goymann and Wingfield 2004). If energetic demands exceed available supplies then individuals may enter a state of allostatic overload, where high levels of glucocorticoids trigger emergency survival mechanisms (Goymann and Wingfield 2004). Allostatic overload induces the suppression of behaviors unrelated to immediate survival. Consequently, individuals often suffer reduced immune function, and increased susceptibility to infection, parasites, and/or disease (Boonstra et al. 2001; Dhabhar and McEwen 1997).

Landys et al. (2006) proposed that glucocorticoids mediate three behavioral and physiological states. Basal levels of glucocorticoids operate only to support metabolism necessary in undisturbed animals at rest (State A, Figure 2). Intermediate glucocorticoid levels are necessary to support the increased demands of predictable life-history events such as migration, onset of winter, and

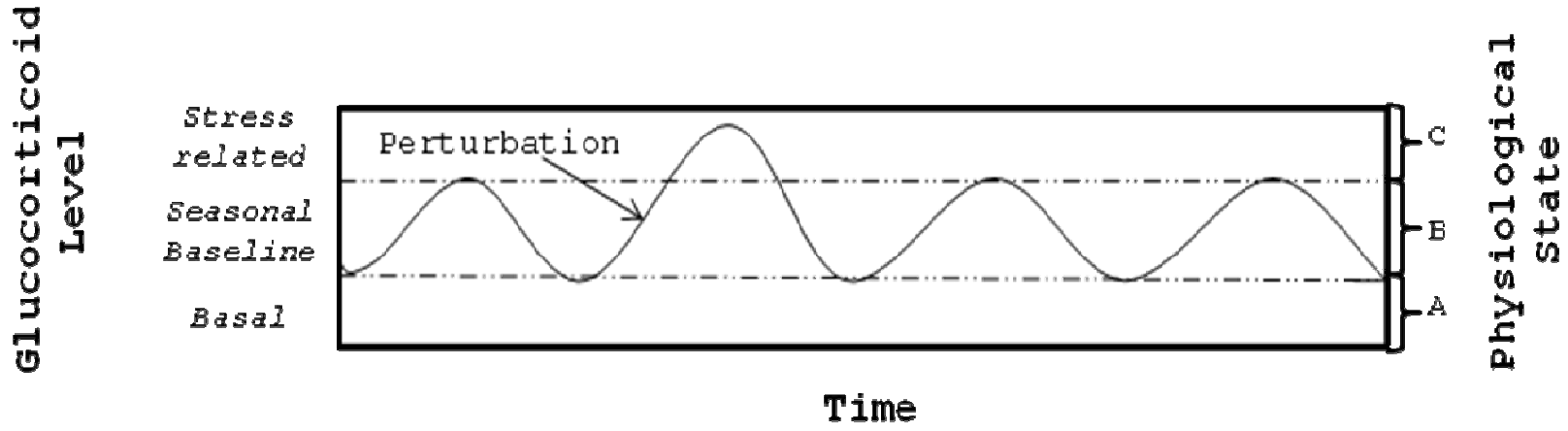

Figure 2. Hypothetical glucocorticoid level at the seasonal baseline and changes in physiological state over time and in response to an environmental or social perturbation. Redrawn from Landys et al. (2006).

breeding. Here, glucocorticoid secretion is presumably associated with cues that indicate the onset of these events rather than a stressor *per se* (State B, Figure 2). By contrast, during life-history emergencies, allostatic load is expected to exceed the immediate ability of animals to cope metabolically (Level C, Landys et al. 2006; Table 1). The allostatic model may be applicable to animals that acquire and defend territories because agonistic interactions may induce acute or chronic stress which influences stress hormone responses. Under this hypothesis, individuals that acquire and maintain territories without high levels of aggression are predicted to have relatively low glucocorticoid levels, whereas, individuals that must fight aggressively or display frequently to acquire territories are expected to have higher glucocorticoid levels (Goymann and Wingfield 2004).

Relationship between Corticosterone and Testosterone

Corticosterone levels sometimes vary seasonally to support periodic demands for energy mobilization associated with the behavioral outcomes of elevated testosterone (Goymann et al. 2007). In cases where social instability becomes chronic, corticosterone levels may surpass a threshold level causing testosterone to decline (reviewed

by Moore and Jessop 2003), an interaction that has been termed the social stress hypothesis (Soto-Gamboa et al. 2005; Table 1). There is some support for the predicted negative influence of chronically elevated corticosterone on testosterone, although the patterns of secretion for these hormones are highly variable across amphibian and reptilian taxa and may be influenced by numerous factors including body condition, environmental conditions, reproductive state, and latitude (reviewed by Moore and Jessop 2003), as well as metabolic concentrations and affinities of binding globulins, mineralcorticoids, or glucocorticoid receptors (Moore and Jessop 2003). Some studies have shown that increased corticosterone levels induced exogenously (Denardo and Licht 1993; Tokarz 1987), or by social conflict (Van Duyse et al. 2004) and handling stress (Manzo et al. 1994; Moore et al. 1991; 2000; 2001) reduced plasma testosterone. By contrast, increased corticosterone production and testosterone levels were not always antagonistic (Klukowski and Nelson 1998; Orchinik et al. 1988), suggesting that the type of social stimulus may play a role in the relationship between these two hormones.

Predictions of Social Stress Hypotheses in Male Collared Lizards

Male collared lizards exhibit a range of behaviors that are likely energetically expensive and vary temporally over the activity season. These include patrol, distant display, male-male encounters, and courtship (all described below; Baird et al. 2001; 2003). In a previous study conducted at Arcadia Lake, Oklahoma the cumulative rate of these behavior patterns was lowest in April and May, at their peak in June, and decreased in July (Baird et al. 2001; 2003). Because glucocorticoid secretion, and in turn allostatic load are expected to increase as a consequence of the high energetic demands associated with these behavior patterns (McEwen and Wingfield 2003), it is reasonable to predict that corticosterone levels in male collared lizards should show a corresponding pattern of temporal variability. Alternatively, if allostatic load is independent of cumulative social demands, then corticosterone levels should remain constant throughout the activity season. I tested these predictions by measuring baseline corticosterone levels in territorial male collared lizards each month throughout the activity season.

The stress response may also be induced during periods of social instability (Soto-Gamboa et al. 2005). The social

stress hypothesis predicts that corticosterone levels should increase to support heightened activity during social encounters (Soto-Gamboa et al. 2005), and as a result testosterone levels may decline (Table 1). Because free-ranging collared lizard males at Arcadia Lake engage in direct aggressive conflict only infrequently, they are not expected to experience high allostatic loads. Therefore, repeated experimental exposure to rivals may provoke an increase in corticosterone as a consequence of high social stress which could result in a subsequent reduction in testosterone. To test this prediction, I repeatedly introduced tethered rivals to resident male collared lizards over a series of days to determine whether or not such social instability prompted an increase in corticosterone and reduction in testosterone.

Methods and Materials

Study site, study organism, and general methods

I conducted this study during April to July in both 2006 and 2007 at the Arcadia Lake dam located 9.6 km east of Edmond, Oklahoma on State Highway 66 (longitude: 97° 21' 47" latitude: 35° 38' 54"). Collared lizards at this site occupy three distinct homogeneous rock habitat patches

(areas 1505-19850 m^2) composed of granite boulders imported to construct flood control spillways. Each site is free from visual obstructions and has restricted human access allowing for observation of lizards with minimal disturbance. Rock patches have been mapped using GIS of reference points arranged in 10 m (± 1.0 m) grids (Baird and Timanus 1998; Baird et al. 2001).

Mark-recapture studies have been conducted on hatchling and adult lizards at this site since 1990, therefore the precise age, sex, and social history of all individuals used in my study were known (Baird et al. 1996; 2001). Upon hatching, all lizards were marked permanently by clipping the terminal phalanges of three digits in unique combinations. For identification from a distance, unique color combinations of temporary, non-toxic acrylic paint dots were applied to the dorsum. Periodically throughout the activity season, I captured adult lizards used in my study by noose to make standard measurements (snout-to-vent length = SVL, ± 1.0 mm; total body mass, ± 1.0 g), and to re-apply paint dots when lizards had molted. All individuals were returned to their original capture location within 5 min of capture to minimize the influence of handling stress on social behavior.

Collared Lizard Social Behavior

At Arcadia Lake, collared lizard males emerging from hibernation for their second activity season (April-late July) usually obtain territories that they defend throughout their lifetimes using high rates of patrol and display, and less frequently by chasing and fighting with other males (Baird et al. 2001; 2003; Baird and Hews 2007). Rates of male display peak in June which is the middle of the activity season, perhaps to ensure access to the same territory the following year without requiring high rates of overt aggression (Baird et al. 2001; 2003). Territorial male collared lizards attempt to monopolize matings with up to ten females that inhabit smaller non-defended home ranges that are overlapped by male territories (Baird et al. 2001; 2003; Baird and Hews 2007). Neither sex provides care of eggs or offspring.

Females that are 2 y and older begin producing their first clutches during April. By contrast, first-year females that are large enough to become reproductively mature do not begin egg production until June, when 2 y and older females are producing their second clutches (Baird et al. 2001; 2003; Baird 2004). The frequency with which territorial males court females increases during these two periods (Baird et al. 2001; 2003).

I measured the frequency of distant displays and proximal encounters. Distant displays are those performed when males are more than 5 m from conspecifics, and the display pattern does not evoke a response from conspecifics (Baird et al. 1996; 2001; 2003; Baird and Hews 2007). Distant displays include vertical movements of the head (head bobs), vertical movements of the torso by flexion of the limbs (pushups), and lateral compression while the torso is elevated and the dewlap extended (full-show). By contrast, proximal encounters between two males involve approaches to within 1 m of each other while initiating one or more of the displays described above, chases, and sometimes attacks which can including biting (Baird et al. 2001; 2003; Baird and Hews 2007). Proximal courtship encounters are characterized by either a male or a female lizard closing to within one body length of the other, the mutual exchange of displays, and one or both lizards making physical contact with their partner which may involve nudging with the snout, superimposition of the torso, limbs and tail, turning circles while maintaining contact, and males grasping females by the neck and mounting them dorsally (Baird et al. 2001; 2003; Baird 2004). For purpose of analysis, I pooled all displays, both distant and

proximal, to determine the total rate of display which I examined statistically.

Within Activity-Season Variation in Hormone Levels

To examine possible variation in testosterone and corticosterone levels throughout the activity season, I sampled blood (see below) from territorial males (n = 15) during the first week of each month (in 2006, April 6-8, May 8-9, June 5-7, July 4-6; in 2007, April 1-2, May 1-9, June 1-2, and July 2-6). The mean time span in days over which blood samples were collected within months was 5.35 ± 0.72 days. This sampling regime allowed measurement of testosterone and corticosterone levels following the emergence of males from hibernation when they were not yet defending territories (early April), during the middle of the breeding season when males were defending territories and females were producing eggs (May and June), and after females had laid their last clutches and male territory defense had diminished (July; Baird et al. 2001; 2003).

Intrusion Experiments

To test the predictions of both the challenge and social stress hypotheses, I recorded behavior using focal observations (Altmann 1974) of territorial males under

different social conditions. Trials lasted four consecutive days and were conducted during May and June of 2006 and 2007 (Table 2). On each of the four days, I recorded the behavior of male subjects for 30 min prior to the introduction of an intruder (baseline phase), for 15 min when the intruder (or an empty noose pole; see below) was present, and then for 30 min immediately following removal of the intruder or noose pole. For analyses (see below), I pooled the 15 min during intrusions and the 30 min observation period immediately following intrusion for each day to compare with baseline rates. During each intrusion, I observed resident males from 10-20 m and recorded by speaking into a tape recorder the occurrence of all distant displays and behavioral acts initiated by the focal male.

Behavioral data recorded on the first day of trials (acute phase), were used to address the predictions of the challenge hypothesis, whereas data recorded on the following three days (chronic phase) were used to address the predictions of the social stress hypothesis (Table 2). I quantified behavior during the baseline phase as the total rate of displays per minute by dividing the total number of behavioral acts initiated during pre-intrusion baseline focal observations on the first day by 30 min. Even though I also recorded a 30 min observation prior to

Experimental Phase			
Baseline		**Acute**	**Chronic**
Day -1	**Day 0**	**Day 1**	**Day 2, 3, & 4**
Pre-Intrusion (30 min)	REST	Pre-Intrusion (30 min)	Pre-Intrusion (30 min)*
		During-Intrusion (15 min)	During-Intrusion (15 min)
		Post-Intrusion (30 min)	Post-Intrusion (30 min)
• Day -1 blood sample		• Day 1 blood sample	• Day 4 blood sample
		Challenge Hypothesis	Social Stress Hypothesis

Table 2. Experimental design for intrusion experiments during May and June in 2006 and 2007. The asterisk indicates chronic-phase intrusion focal observations not used in analyses.

intrusions on the second through the fourth days, I did not use these data in analyses because they might not be indicative of baseline levels of behavior because males had interacted with intruders on previous days. I quantified behavior during and following acute-phase intrusions as the total number of total displays initiated during (15 min) and following (30 min) intrusions by the sum of both observation periods (45 min). I quantified behavior for the chronic-phase intrusions by dividing the cumulative number of behavioral acts observed during and following intrusions on the second through fourth days by the total observation time (45 min x 3 days = 135 min).

I collected blood samples at three time periods to test the challenge and social stress hypotheses. I collected the first blood sample from resident and control males 3 d prior to the first intrusions (baseline phase), and then a second sample following the acute-phase intrusion to test the hypothesis that there should be no change in testosterone as a consequence of acute social challenges. I then collected a third sample following the focal observation of the chronic phase to test the effects of chronic challenge on testosterone, corticosterone and the relationship between the two hormones.

Intrusions were conducted between 0800–1500 h when substrate temperatures were 30-38°C, a range over which frequencies of social behavior are independent of substrate temperature in males of this species (Baird et al. 2001). Intruders were tethered on 10 cm of monofilament line attached to the tip of a 3 m long pole to limit their movement (Klukowski and Nelson 1998). I placed intruders 2-3 m from and in clear sight of defenders, and began timing trials only when intruders had been placed on the substrate. If the defender did not respond after 1 min, I moved the intruder increasingly closer (0.5 m intervals up to 0.5 m away) to ensure that I provoked an aggressive

response ($\bar{x}$ moves/intrusion = 2.5). In trials when aggression escalated to prolonged biting, to avoid injury to intruders, I moved them away for 30-60 s and then replaced them 1 m from residents ($\bar{x}$ interruptions/intrusion = 0.14).

To control for possible fluctuations in hormone and behavior resulting from factors other than interactions with intruders, for each experimental trial I conducted a simultaneous trial on a different male by placing an empty noose-pole 2-3 m away from him. I chose males for control treatments (n = 12) to match the age and size (± 5 mm SVL) of challenged males. Each time that control males moved more than 5 m away from the noose pole, I moved the tip of the pole to within 2-3 m of the male. I recorded the same behavioral data for control males as described above for challenged males.

Because prior wins or losses during contests with familiar opponents may alter male hormonal (Oyegbile and Marler 2005) and behavioral (Fox and Baird 1992; Husak and Fox 2003) responses to aggression, I captured intruders (n = 15) away from the Arcadia Lake site along roadways 3.2 km north of Arcadia, Oklahoma and at Lake Thunderbird Dam in Norman, Oklahoma (longitude: 97° 13' 5", latitude: 35° 13' 15") to ensure that challenged males had no previous

experience with intruder males. Ten of the intruder males were returned to their original capture locations unharmed. Four males were sacrificed and placed in the University of Central Oklahoma Natural History Museum, Herpetology collection (UCONHM 1273-1276), and one intruder male escaped at Arcadia Lake and was not re-sighted.

Blood Sampling

Blood was collected by rupturing the post-orbital sinus with a heparinized microcapillary tube. Blood samples were collected from 0800-1400 h, over which hormone levels do not show significant temporal fluctuations (Baird and Hews 2007). I captured lizards within 5 min of the onset of pursuit, and collected blood within 1 min of capture, a total amount of disturbance time that does not influence hormone levels in males at this site (Baird and Hews 2007). After blood collection, males were immediately released at their capture locations.

Blood samples were held on wet ice in the field for a maximum of 3 h and then transported to the laboratory at the University of Central Oklahoma. Once in the laboratory, I immediately centrifuged samples and separated off the plasma, which was stored at -80^{o}C. In August 2007, samples were packed in dry ice and transported overnight to Indiana

State University where radioimmunoassays were run following the methods of Wingfield and Farner (1975), adapted for use in collared lizards (Baird and Hews 2007).

Radioimmunoassays

Plasma samples (20 µl) were equilibrated overnight with 2400 cpm of each titrated steroid for determining sample recoveries. Steroids were extracted from the plasma samples twice with diethyl ether, the ether phase was dried under nitrogen gas, steroids were resuspended in 10% ethyl acetate in isooctane, and then refrigerated overnight. For chromatographic separation of steroids, extracted samples were added to minicolumns (celite: propanediol: ethylene glycol, 4:1:1 w: v: v over celite: water, 3:1 w: v). Neutral lipids, testosterone, and corticosterone were eluted from the columns with 0%, 10%, 20% or 52% ethyl acetate in isooctane (two discards, T, and B fractions, respectively). Following evaporation of organic solvents, I re-suspended steroids in phosphate buffered saline with gelatin and measured plasma levels of the two hormones using a modification (Baird and Hews 2007) of the radioimmunoassay procedure of Wingfield & Farner (1975). Duplicate sample aliquots were incubated overnight with antibodies (testosterone, WLI-T3003, RDI Division of

Fitzgerald Industries Int., Concord, MA, used for T fractions; corticosterone, B3-163, Esoterix Inc., Calabasas Hills, CA) and titrated steroids (New England Nuclear NET 553 and NET 399). Unbound steroids were removed by adding dextran-coated charcoal and centrifugation. Radioactivity (cpm) was measured following addition of scintillation fluid to the supernatant and a 12 h hold. All samples were run in three assays. Calculations of steroid concentrations were corrected for plasma volumes and individual recoveries. Use of the radioimmunoassay was validated by Baird and Hews (2007).

Statistical Analyses

For all analyses where values did not meet the assumptions for parametric statistics, normality was achieved using log base 10 transformations. In the case of significant interaction terms where main effects were significant, we calculated simple effects (Kuehl 2000). All analyses were run using SPSS 18.0 and significant values reported at $\alpha < 0.05$. Fisher's PLSD post hoc analyses were used to determine specific differences between independent variables.

To determine baseline levels of testosterone and corticosterone during April–July, I calculated monthly

means and used repeated measures ANOVA with month as the repeated factor and testosterone and corticosterone concentrations (ng/mL) as dependent variables (separate ANOVA's, n = 15).

To analyze changes in testosterone and corticosterone concentrations as consequences of acute and of chronic exposure to territorial intruders, I used separate repeated measures ANCOVA's with treatment (challenged versus control) as the independent variable, and hormone levels measured during acute- and chronic phases as the repeated factors. To control for any hormonal differences between individuals at the start of the study, I included baseline testosterone and corticosterone levels as covariates in these analyses.

I analyzed changes in frequencies of male behavior (prior to versus following challenges) using repeated measures ANCOVA. The dependent variable was the total number of behavioral acts initiated per min during each of the three phases (repeated factor): acute phase pre-intrusion, acute phase during plus post-intrusion, and chronic phase during plus post-intrusion (Table 2). The independent variable was treatment (challenge versus control). Because in previous studies male social behavior varied significantly over the activity season (Baird et al.

2001; 2003), we used the beginning date of each trial (in ordinal form) as the covariate in this analysis.

I also evaluated potential relationships between hormone levels and behavior, using Box's M homogeneity of variances test (Timm 2002). Variables included in this analysis were the total acts initiated during intra- and intersexual encounters, total distant displays, and the differences in testosterone and corticosterone levels between the baseline phase and the post-intrusion period of the acute phase, and between the baseline phase and the post-intrusion period of the chronic phase. I used the overall statistic, Box's M, to compare the covariance structure in challenged versus control males.

Results

Behavioral Responses to Acute and to Chronic Challenges

My sample for behavioral observations (challenged males, n = 10, control males, n = 12) is smaller than that for hormonal measurements (challenged males, n = 15, control males, n = 16) because I inadvertently recorded over several records before I had transcribed them. The frequency of total displays exhibited by challenged and control males was not significantly different across phase ($F_{2, 38}$ = 2.63, p = 0.09). However, within subjects, there

was a significant phase x date interaction ($F_{2, 38} = 3.55$, $p = 0.04$), suggesting that the effects of experimental phase were not the same across the activity season. The phase x treatment interaction ($F_{2, 38} = 15.64$, $p = 0.07$) approached statistical significance, indicating that the effects of experimental phase may have differed for challenge versus control males. Thus, to determine how date and treatment influenced the frequency of total display across experimental phase, I conducted separate repeated measures ANOVA's on the frequency of total displays for trials in May (1-31) and June (1-30).

For trials conducted in May, display frequency did not differ between challenged and control males ($F_{1, 8} = 0.81$, $p = 0.39$) during baseline-, acute-, or chronic-intrusion phases, and there were no significant interactive effects (Figure 3A). Challenged and control males during June trials displayed with similar frequencies ($F_{2, 16} = 0.86$, $p = 0.44$) during each of the three phases (Figure 3B). However, there was a significant within-subjects interaction of phase x treatment ($F_{2, 16} = 4.87$, $p = 0.02$). Therefore, to further investigate the relationship between phase and treatment on the frequency of total display during June trials, I conducted separate repeated measures ANOVA's for challenged and control males.

For control males, there was no within-subjects effect of phase ($F_{2, 9} = 0.10$, $p = 0.90$). However, as expected, challenged males displayed at significantly different ($F_{2, 7} = 3.62$, $p = 0.05$) rates during the three phases (Figure 3B). Pairwise comparisons revealed that challenged males performed more displays during both acute- ($p = 0.001$) and chronic-intrusion ($p = 0.008$) phases than during the baseline phase, but displayed at similar rates ($p = 0.53$) during acute- and chronic phases (Figure 3B).

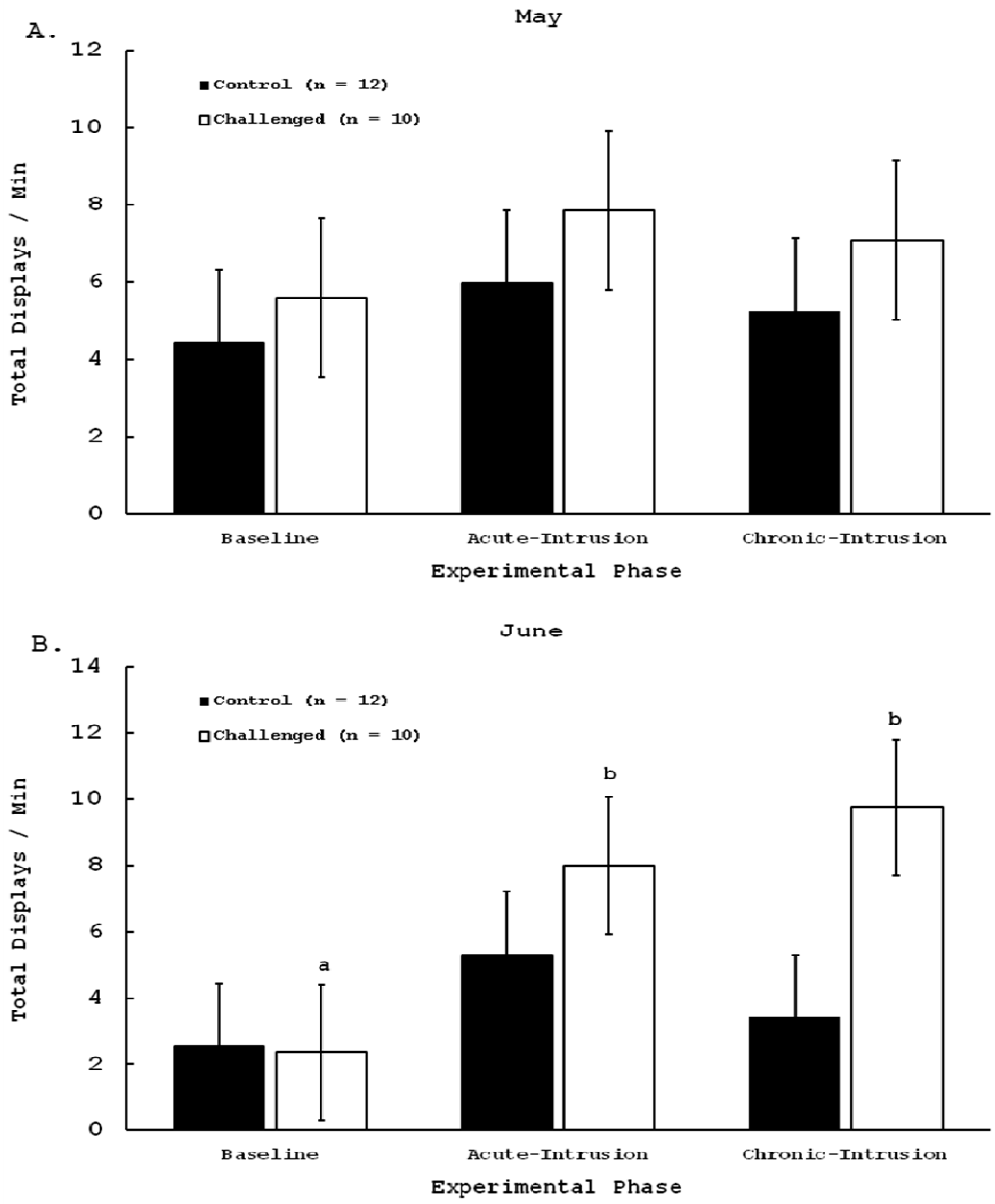

Figure 3. Total number of displays/min (mean ± 1.0 *SE*) during baseline-, acute-, and chronic phases for challenged and control males during May (A) and June (B). In June, different letters indicate significant differences between acute- (p = 0.01) and chronic phases (p = 0.01) compared with the baseline phase.

Within Activity-Season Variation in Hormone Levels

Mean testosterone levels varied ($F_{3, 42} = 3.93$, $p = 0.02$) from April to July (Figure 4A). Testosterone levels were similar and highest in April and June ($p = 0.46$), and similar and lower in May and July ($p = 0.86$). April testosterone levels were not statistically different ($p = 0.06$) from May (Figure 4A). In contrast with testosterone, corticosterone levels remained stable ($F_{3, 42} = 0.37$, $p = 0.78$) throughout the activity season (Figure 4B).

Testosterone and Corticosterone Relationships

I assessed relationships between testosterone and corticosterone by calculating Pearson's Product-moment correlation coefficients for baseline-, acute-, and chronic phases for challenged and control males. Coefficients were not statistically significant for either control (baseline phase, $r = 0.30$, $p = 0.26$; acute phase, $r = 0.35$, $p = 0.18$; chronic phase, $r = -0.22$, $p = 0.43$), or challenged males (baseline phase, $r = -0.48$, $p = 0.07$; acute phase, $r = 0.24$, $p = 0.39$; chronic phase, $r = 0.22$, $p = 0.44$; Figure 5A, B, and C). Because there was no indication that testosterone and corticosterone values were correlated, in subsequent analyses I considered each hormone separately.

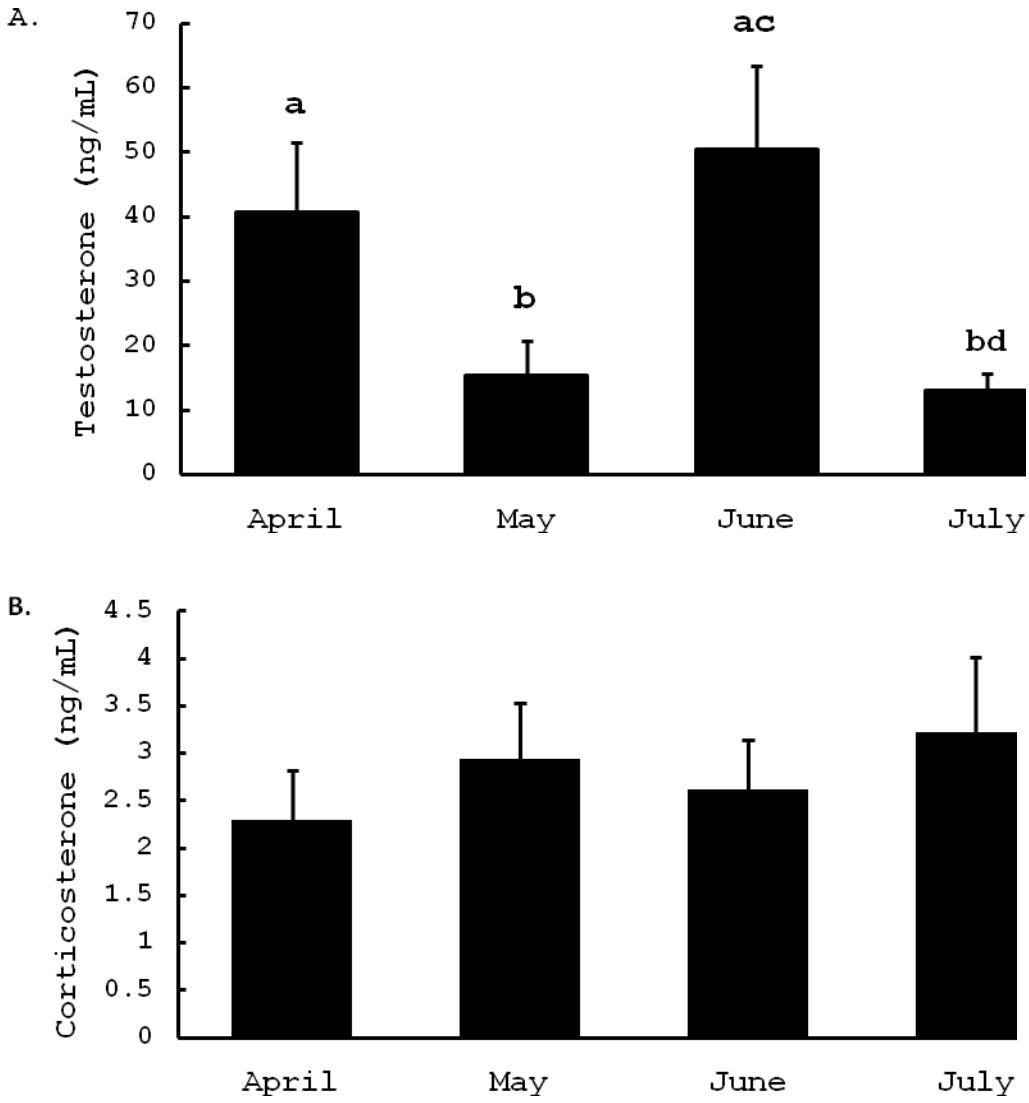

Figure 4. Mean (± 1.0 SE) testosterone (A) and corticosterone (B) concentrations in the same territorial male collared lizards (*n* = 15) from April to July of 2006 and 2007. Different letters indicate significant differences in testosterone between months in a repeated measures ANOVA.

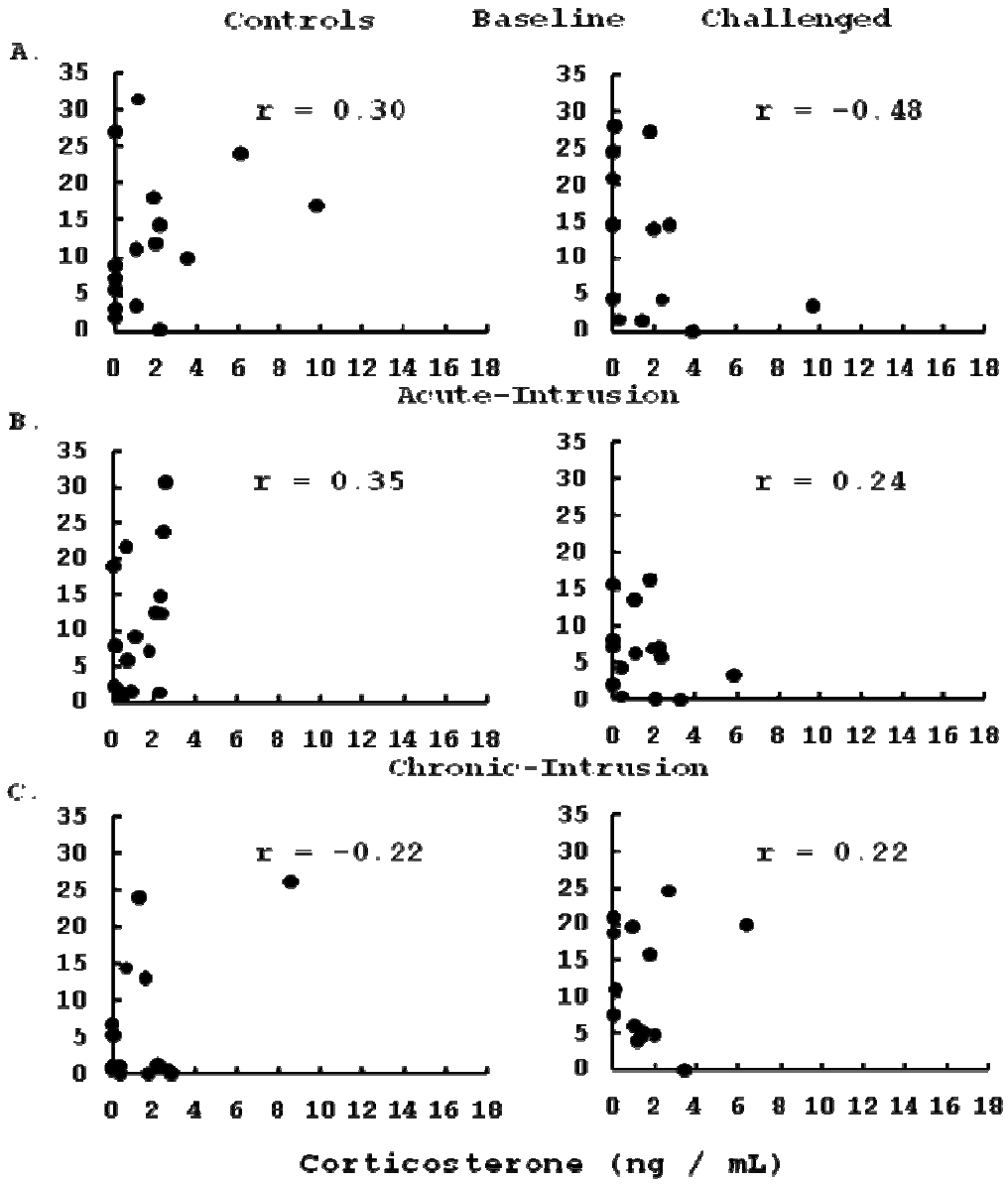

Figure 5. Levels of testosterone graphed against levels of corticosterone in control (left panel) and challenged (right panel) territorial males during (A) baseline-, (B) acute-, and (C) chronic-intrusions.

Hormonal Responsiveness to Acute and Chronic Challenges

Repeated measures ANOVA comparing testosterone during baseline-, acute-, and chronic phases revealed that neither the between-subjects effect ($F_{1, 29} = 0.36$, $p = 0.55$), nor the within-subjects main effect of phase ($F_{2, 58} = 2.17$, $p = 0.12$) were statistically significant (Figure 6A). The within-subjects phase x treatment interaction was significant ($F_{2, 58} = 3.10$, $p = 0.05$, Huynh- Feldt $p = 0.05$), and this interaction term increased ($p = 0.01$) after discarding one outlier: a control male having a very high baseline testosterone value (89 ng/mL) when this same male had a non-detectable level for the third blood sample following the chronic-intrusion phase.

We examined the effects of phase, for each treatment group for several reasons. First, polynomial contrasts revealed no significant linear effects of phase ($F_{1, 29} = 2.30$, $p = 0.10$), or a phase x treatment interaction ($F_{1, 29} = 1.78$, $p = 0.19$), however there was a significant quadratic contrast ($F_{1, 29} = 5.15$, $p = 0.03$). Furthermore, the significant ($p = 0.05$) within-subjects phase x treatment interaction combined with the significant quadratic contrast also argued for examining main effects of phase for each treatment group separately. During intrusion experiments, testosterone varied significantly with phase

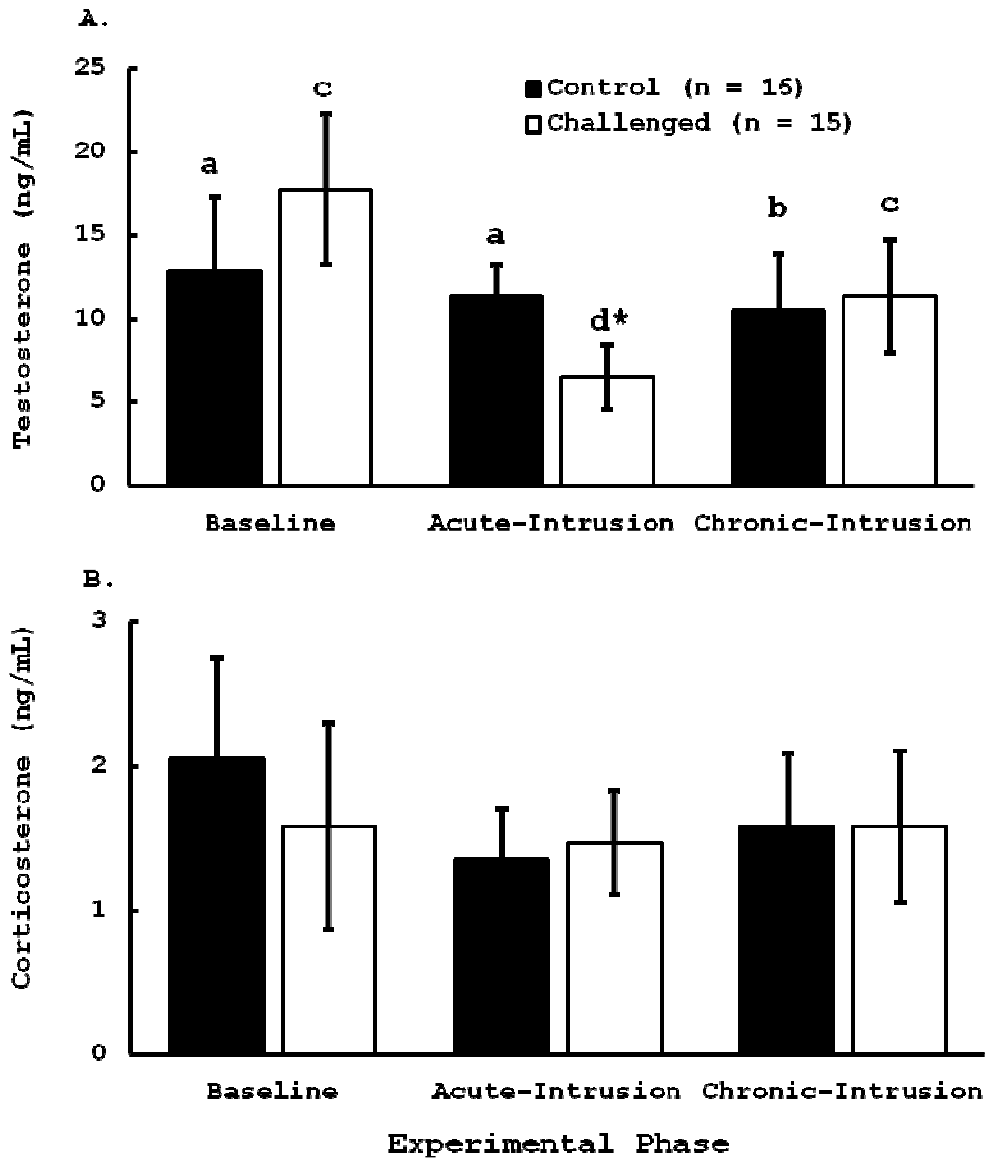

Figure 6. Mean (± 1.0 S*E*) testosterone (A) and corticosterone (B) concentrations during baseline-, acute-, and chronic-intrusion phases for challenged (open bars) and control males (dark bars). Different letters indicate a significant decrease in testosterone from baseline- to the chronic-intrusion phase in control males in a repeated measures ANOVA. The asterisk indicates a marginally significant decrease.

in control males. The within-subjects main effect for phase was not significant ($F_{2, 30}$ = 3.12, p = 0.06; Huynh- Feldt p = 0.06), however the polynomial test for first order (linear) effects for phase was significant ($F_{1, 15}$ = 5.05, p = 0.04), whereas the quadratic contrast was not (p = 0.41). Mean testosterone concentrations following the acute phase were similar to the baseline phase (p = 0.53), however, mean testosterone level for control males showed a significant linear decrease from the baseline phase to the chronic phase.

During intrusions, testosterone levels varied across phase in challenged males (Figure 6B) but in a different pattern than for controls. Although there was no significant within-subjects effect of phase ($F_{2, 28}$ = 2.15, p = 0.14), there was a significant quadratic component to the polynomial contrasts ($F_{1, 14}$ = 6.40, p = 0.02). This indicated that testosterone levels in challenged males did not show a simple linear decrease but instead decreased following acute-phase intrusions, followed by an increase after chronic-phase intrusions (Figure 6A). Post-hoc comparisons suggested a trend (p = 0.07) for testosterone levels to decline between baseline and acute phases for challenged males, whereas testosterone levels did not

differ significantly (p = 0.80) between these two phases (Figure 6B), and there were no significant differences among phase for either treatment group. Between-subjects effects of treatment ($F_{1, 27}$ = 0.17, p = 0.68), within-subjects effects of phase ($F_{2, 54}$ = 0.01, p = 0.10), and the within-subjects phase x treatment ($F_{2, 54}$ = 0.37, p = 0.69) interaction were all not statistically significant (Figure 6B). Similarly, neither polynomial contrasts for linear (p values > 0.40) nor quadratic (p values > 0.91) main effects or their interaction terms were statistically significant.

Box's M test comparing correlations among behavioral and hormonal variables in control versus challenged males revealed a significant difference (M = 110.95, $F_{28, 788}$ = 1.96, p = 0.002) between the correlation matrices of males in these two treatment groups. However, the small sample for behavioral variables precluded my examining which variable combinations differed in the two treatment groups.

Discussion

Androgen Responses to Social Challenges

According to the predictions of the challenge hypothesis, territorial male collared lizards should have maintained testosterone levels close to the physiological maximum throughout the entire activity season. Instead,

testosterone levels in these males showed significant within-season variation. Despite variable levels of testosterone across the activity season, male collared lizards did not show an androgen response to staged social challenges. The lack of androgen responsiveness to intruders during May and June trials cannot be attributed to the inability of males to increase testosterone. Even though baseline testosterone levels from a pooled sample of males during June were higher than those in May, challenged levels of testosterone during these two months (Figure 6) were only approximately 12 ng/mL compared with 40-50 ng/mL at baseline for all males in June. This result reveals that males used in experiments were not at physiological maximum for testosterone, and hence could have responded to challenges if in fact testosterone and aggression were correlated. Previous studies on Oklahoma collared lizards have yielded similar results. Blood samples collected from Arcadia Lake males in early June (Baird and Hews 2007) revealed testosterone levels that were approximately one-half the levels in my study. Furthermore, Baird and Hews (2007) showed that testosterone levels were similar in territorial and non-territorial males, and there was no correlation between testosterone and rates of distant display, patrol, male-male, or courtship encounters.

Testosterone levels in late May were also not correlated with male territory area at Sooner Dam, Oklahoma (Husak et al. 2006) which is a potential measure of social dominance. Taken together, there is no evidence that agonistic social interaction prompts androgen secretion in collared lizards, which is consistent with the meta-analysis findings on numerous other vertebrates (Hirschenhauser and Oliveira 2006).

Rather than increase testosterone, males in my study exhibited a non-significant marginal decrease in response to challenges from same-sex rivals even though aggressive behavior increased in intensity and frequency. One possible explanation for this is threat of losing social dominance (Maner et al. 2008; Mehta and Josephs 2006). During encounters between free-ranging collared lizards, both participants have the option to retreat, which may be an important signal of winning or losing encounters. In my study, intruder males were tethered, and hence could not retreat. Moreover, I moved intruders closer in some trials. As a consequence of my experimental protocol, residents may have reacted physiologically as if they had lost, or at least did not win decisively, which could potentially decrease testosterone levels.

The observed decrease in testosterone post-challenge may also have resulted from decreased courtship activity during social challenges. During and following challenges, males engaged in courtship encounters at approximately one-half the rate observed prior to intrusions. Reduced testosterone has been observed in response to the temporary suspension of courtship activities during challenges in other species (Santangelo et al. 2002; Wong 2004). Taken together, the results of my study support the hypothesis that testosterone secretion in male collared lizards is stimulated by interactions with receptive mates. Using data from a previous study showing within-season variation in male display and courtship behavior at this site (Baird et al. 2003) it is interesting to compare within-season variation in behavior patterns with that for testosterone. Testosterone levels were high in April when males were courting older females that became receptive earliest, but were even higher in June when males were courting both females that were receptive for their second clutches and first-year females that were receptive for their first clutches (Baird et al. 2001). Regulation of testosterone to coincide with female receptivity could involve a number of mechanisms including variable sensitivity of the pituitary to gonadotropin releasing hormone, variable sensitivity of

the testes to lutenizing hormone, or regulation of testosterone receptor sensitivity at specific target tissues (Adkins-Regan 2005; Becker et al. 2002; Jawor et al. 2006). By whatever means, males that regulate testosterone levels to correspond with peak female receptivity would avoid the potential costs that can be associated with maintaining high testosterone levels throughout the entire season (Wingfield et al. 2001). Costs of maintaining chronically high testosterone levels may be decreased by reducing sensitivity to this hormone when it is not necessary to promote fitness (Adkins-Reagan 2005). Such a regulatory mechanism that reduces costs may be particularly advantageous in species such as *C. collaris* that continue to defend territories for several seasons (Baird et al. 2003; Schwartz et al. 2007).

Does Intra- and Intersexual Activity Influence Corticosterone?

Even though male collared lizards exhibited markedly different behavior patterns over their activity season, corticosterone levels remained constant throughout. This pattern does not support the prediction that certain behaviors are more energetically expensive than others and hence produce more stress (Goymann and Wingfield 2004).

There was also no support for the prediction that exposure to a novel stressor stimulates increases in corticosterone, because corticosterone levels did not differ among baseline-, acute-, or chronic-intrusion phases during challenge trials. One possible explanation for the lack of a hormonal stress response is that conspecific intruders were not perceived to be a substantial threat, perhaps because intruder males behaved submissively by laying flat. Another possible explanation is that I did not measure the most biologically relevant form of corticosterone. I measured total plasma corticosterone levels which consists of both bound (Glucocorticoid binding globulin complexes) and unbound (free) corticosterone. Because free corticosterone is released from glucocorticoid binding globulin complexes during periods of high energetic demand and is quickly cleared from the body (Breuner and Orchinik 2002), it is possible that even small changes in free corticosterone were masked by including the bound fraction.

Conclusions

Inconsistent with the challenge hypothesis, male collared lizards exhibited significant variation in testosterone levels over the breeding season. Despite marked increases in the frequency and intensity of display,

at least during June, challenges did not illicit an increase in testosterone even though baseline levels were not maximal. Peaks in testosterone occurred during April and June when male courtship and display were at their highest (respectively). Thus, the results of my study suggest that testosterone and aggression are unrelated, but that courtship activity may influence seasonal changes in testosterone, results that are consistent with meta-analysis of similar studies on a large, phyletically diverse sample (Hirschenhauser and Oliveira 2006).

Inconsistent with the social stress hypothesis, corticosterone levels did not change during the activity season or following acute or chronic exposure to conspecific challengers. Furthermore, corticosterone levels were not elevated enough to cause decreases in testosterone as a consequence of potentially antagonistic activity between these two hormones. One possibility for the lack of a stress response to challengers may be that intruder male were not perceived to be a threat. Another possibility may be that I may not have measured the most biologically relevant form of corticosterone.

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