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ENDOCRINE MEDIATION OF ALTERNATIVE MATING TACTICS IN TOADS: A PROXIMATE PERSPECTIVE ON ECOLOGICAL PATTERNS AND SEXUAL SELECTION

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A Dissertation APPROVED for the
DEPARTMENT OF ZOOLOGY

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Preface

The first chapter of this dissertation has been formatted for the journal Behavioral Ecology, the second for Hormones and Behavior, and the third for the Proceedings of the Royal Society of London, Series B.

Abstract

Male Great Plains toads (*Bufo cognatus*) and Woodhouse's toads (*Bufo woodhousii*) alternate between one of two mating tactics; they may either vocalize to attract mates or they may adopt a non-calling "satellite" tactic. Alternation from the calling to the satellite tactic is associated with a reduction in body mass during chorus activity that drives an increase in circulating corticosterone levels. Elevation in corticosterone levels result in a progressive decrease in call duration that eventually leads to the cessation of calling activity and the expression of the alternative non-calling satellite tactic. Females prefer the calls characteristic of males with the lowest corticosterone levels, suggesting that selection may favor any heritable component associated with maintenance of relatively low corticosterone levels or suppression of the stress response. However, sexually-parasitic males practicing alternative reproductive tactics also prefer to associate with calling males with the lowest corticosterone levels that produce the longest calls. Hence, although calling males in the best physical condition with the lowest levels of corticosterone produce the most attractive calls, males with the longest calls may also experience a reduction in reproductive success by attracting sexually parasitic conspecific satellite males. Therefore, directional selection on preferred male traits via female choice may be countered by host selection via males practicing alternative mating tactics.

Chapter I

Steroid Hormone Profiles and Relative Body Condition of Calling and Satellite Toads: Implications for Proximate Regulation of Behavior in Anurans

ABSTRACT -- Males of most anuran species (frogs and toads) vocalize to attract mates. However, individuals of many vocal species may also adopt alternative non-calling “satellite” tactics. Satellite males characteristically remain in close proximity to calling conspecifics and attempt to intercept incoming females attracted to advertising males. Emerson (2001) proposed that alternation between calling and non-calling behavior in anurans is mediated by a reciprocal interaction between circulating levels of corticosterone and androgens that is driven by depletion of energy reserves during vocalization. We tested this hypothesis by examining steroid hormone profiles and the relative body condition of calling and satellite Woodhouse’s toads (*Bufo woodhousii*) and Great Plains toads (*B. cognatus*). Consistent with Emerson's hypothesis, callers had significantly higher circulating corticosterone levels and were in better condition than satellites. However, levels of testosterone and dihydrotestosterone did not differ significantly between satellites and callers, and we found no evidence that high levels of corticosterone had an inhibitory effect on androgen production in either species. These data thus support a relationship between corticosterone levels and depletion of energy reserves during bouts of vocalization but suggest that alternation between calling and satellite behavior may be associated with direct effects of corticosterone on brain vocal control centers. We propose a model that incorporates relationships among energy

reserves, androgens, corticosterone, and arginine vasotocin-producing neurons in the telencephalon to explain transitions between calling and satellite tactics in toads.

INTRODUCTION

Alternative mating behaviors occur in all major radiations of vertebrates (Gross, 1986; Rubenstein, 1980), but relatively few empirical data are available regarding underlying mechanisms associated with behavioral expression for most taxonomic groups. Proximate regulation of alternative reproductive tactics in fishes (Brantley et al., 1993; Oliveira et al., 2001; Uglem et al., 2002) and reptiles (Crews 1998; Moore, 1991; Moore et al., 1998; Rhen and Crews, 2002) have been investigated most extensively, and current models are largely based on these organisms. However, many taxa are likely to exhibit differences in proximate regulation of behavior at some level given the disparate neural pathways associated with signal production. For instance, many fishes and squamate reptiles produce visual signals to attract mates whereas most anuran amphibians (frogs and toads) and birds produce acoustic signals.

Alternative mating tactics are particularly pervasive in anurans (Waltz, 1982). Numerous investigations report the existence of non-vocalizing individuals (termed “satellites”) that attempt to intercept incoming females attracted to the vocalizations of calling males (reviewed by Halliday and Tejedo, 1995). In most cases, individuals reportedly alternate between calling and satellite tactics within and/or between breeding bouts indicating that the behaviors are condition-dependent strategies within genetically monomorphic males (sensu Gross, 1986).

Condition-dependent plastic adult phenotypes should be subject to activational effects of hormones that mediate behavioral expression (Moore, 1991; Moore et al., 1998). In anuran amphibians (and birds), production of vocalizations is usually mediated by androgens (Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001; Kelley, 1980; Moore, 1987; Wetzel and Kelley, 1983; Wingfield and Moore, 1987). Current understanding of the relationship between circulating steroid hormones and the expression of alternative mating tactics in anurans is largely based upon the work of Mendonça et al. (1985), who found that satellite male bullfrogs (*Rana catesbeiana*) possessed significantly higher androgens (testosterone + 5α-dihydrotestosterone) relative to calling conspecifics. Mendonça et al. (1985) proposed that lower androgen levels in calling males resulted from stress responses (i.e., interactions between glucocorticoids and androgen-producing cells) associated with territorial defense (male bullfrogs aggressively defend territories and frequently engage in combat, Howard, 1978, 1984).

Vocal production in anurans is one of the most energetically expensive behaviors known for any ectotherm vertebrate, frequently elevating metabolic rates 20 times above basal rates (Wells, 2001). The energetic expenditure associated with vocal production combined with the common inhibition of androgen production associated with elevated glucocorticoid levels (Greenberg and Wingfield, 1987; Knapp and Moore, 1997; Licht et al., 1983; Orchinik et al., 1988; Wingfield and Ramenofsky, 1999) provided the impetus for the Energetics–Hormone Vocalization Model (Emerson, 2001). This model is an extension of the Challenge Hypothesis (Wingfield et al., 1990) and proposes an interaction between corticosterone and androgens to explain transitions between calling and non-calling behavior in anurans. It predicts that the energetic demands imposed by vocal production will elevate circulating corticosterone, which, in turn, reaches a threshold level that inhibits androgen production. At this stage, a male ceases calling until energetic deficits are restored, circulating corticosterone is metabolized, and elevations in androgens mediate another bout of calling.

We tested predictions of the Energetics–Hormone Vocalization Model in two anuran species with different satellite strategies. Non-calling satellite Woodhouse’s toads (*Bufo woodhousii*) characteristically reside along the periphery of the chorus and actively amplex incoming individuals in an attempt to locate females (Sullivan, 1989). Individual *B. woodhousii* periodically alternate between calling and satellite tactics within and/or between breeding bouts (Sullivan, 1989). In contrast, Great Plains toads (*Bufo cognatus*) exhibit a satellite–host association whereby one or more non-calling satellites parasitize a single vocalizing individual male (Krupa, 1989; Sullivan, 1982b, 1983). Satellite males remain stationary, usually within 0.5 meters of their “host”, and also commonly alternate between calling and non-calling tactics (Krupa, 1989; Sullivan, 1982b, 1983).

Individuals of territorial and/or aggressive species may prevent conspecifics from exhibiting particular behaviors irrespective of their physiological condition. *Bufo woodhousii* and *B. cognatus* do not exhibit territoriality in the sense of long-term site fidelity and neither species engages in combat or prolonged territorial bouts (Krupa, 1989; Sullivan, 1982a, 1982b, 1983; Sullivan and Leek, 1986), as does *R. catesbeiana* (Howard, 1978). Although aggressive behaviors among males were reported for prolonged breeding populations of *B. woodhousii* in the desert southwest U.S. (Sullivan, 1982a), aggression has not been observed in explosive breeding populations in the central U.S. Great Plains (B.K. Sullivan, pers. comm.; CJL, pers. obs.). Hence, these non-territorial, non-aggressive toad species were considered appropriate subjects to investigate proximate regulation of behavior because investigations are not confounded by social factors that potentially affect phenotypic expression. We focus on the relationship among circulating corticosterone, androgens, and relative body condition in an attempt to elucidate the factors regulating the expression of plastic adult phenotypes.

METHODS

Field Methods

Our study pond for *B. woodhousii* is located on a privately owned ranch in Norman, Oklahoma, where as many as 500 individuals may gather during the breeding season. *Bufo woodhousii* typically begin breeding following early spring rains (~April). We studied 2 *B. cognatus* breeding sites within 0.5 km of each other (also in Norman, Oklahoma) where a total of ~250 individuals engaged in breeding activity. Breeding activity for *B. cognatus* may or may not overlap with *B. woodhousii* depending on weather conditions (heavy rainfall is required to initiate breeding activity in *B. cognatus*). Both species breed in ephemeral bodies of water in the same area but *B. woodhousii* are largely allotopic to *B. cognatus*.

We recorded behavior of individuals under natural conditions for 10 to 15 min prior to collecting blood samples for hormone analyses. Observations were made with headlamps and/or flashlights and did not have any overt effects on behavior (see also Sullivan, 1983, 1989). In the time frame we used to assess behavioral expression, it was common to observe non-calling satellite males attempting to intercept incoming females and/or nearby swimming males (Krupa, 1989; Sullivan, 1983, 1989). Immediately following focal observations, blood was obtained in the field by cardiac puncture with a heparinized hypodermic needle. Bleeding procedures were completed in < 3 min after hand capture. Blood samples (~ 200 μl) were collected from 112 *B. woodhousii* during breeding activity on 3-5, 8, 9, 13 April and 5, 19 May 2001. Blood samples were collected from a total of 45 *B. cognatus* on 20, 28-31 May and 1 June 2001. These dates largely encompassed the entire breeding season for both species.

Blood sampling took place during peak activity periods from approximately 2300 to 0100 hrs for *B. woodhousii* and from 2200 to 2400 hrs for *B. cognatus*. Both satellites and callers were sampled throughout observed activity periods. When satellites were sampled, a nearby caller was also sampled at the same time, but more blood samples were obtained for callers because they were more abundant than satellites and, therefore, blood sampling was not always paired. Approximately 54% of all data points for *B. woodhousii* consisted of satellites and callers that had blood collected in a paired fashion; 84% of all *B. cognatus* data points consisted of caller–satellite associations that were sampled in a paired fashion. Following blood collection, all individuals were weighed to the nearest 1.0 g with a portable OHAUS digital scale, measured from tip of snout to end of ischium to the nearest 1.0 mm, and numerically marked in sequence on the venter with a portable tattoo device (Tattoo-A-Pet, Inc., Fort Lauderdale, FL) for future identification. Procedures had no overt effects on subsequent behavior; non-satellite males typically resumed calling immediately following data collection and release. Blood samples obtained in the field were kept on ice until they were returned to the lab (< 4 hours) and centrifuged for 12 min at 3000 rpm. Plasma was collected and stored at -20°C until it was assayed for steroid hormones. All procedures were approved by the University of Oklahoma Animal Care and Use Committee (#A3240-01).

Hormone Assays

Plasma samples were extracted with diethyl ether, dried under nitrogen gas, and resuspended in 10% ethyl acetate in iso-octane. Column chromatography was then used to separate dihydrotestosterone (DHT), testosterone (T), and corticosterone (B). Hormone extraction, chromatography, and radioimmunoassay followed Harvey et al. (1997) and Knapp et al. (1999). Testosterone antibody was obtained from Research Diagnostics (Flanders, NJ) and used for both T and DHT assays. Corticosterone antibody was purchased from ICN Laboratories (Costa Mesa, CA).

Plasma samples were analyzed for androgen and B levels in 4 assay runs for *B. woodhousii* and 1 assay run for *B. cognatus*. Mean intra-assay coefficients of variation for *B. woodhousii* for DHT, T, and B were 22.9%, 8.6%, and 5.4%, respectively, based on 2-4 standards run with each assay. Inter-assay coefficients of variation for *B. woodhousii* for DHT, T, and B were 26.1%, 5.5%, and 12.2%, respectively. Intra-assay coefficients of variation for *B. cognatus* for DHT, T, and B were 14.9%, 9.2, and 11.5%, respectively, based on 4 standards run in the single assay. Although the extraction and chromatography procedures remove potential sources of non-specific binding, we validated our assay procedures by verifying that curves generated by serial dilution of a plasma sample with known high hormone levels and from hormone-spiked charcoal-stripped plasma were parallel to the standard curves for each hormone (data not shown). Steroid hormone profiles for callers and satellites were analyzed separately for both species using ANOVA.

Body Condition

To determine if there was evidence of depleted energy reserves for satellites versus callers, the body mass of individuals was examined using ANCOVA with snout-ischial length as the covariate. Residual regression analyses were also performed to examine the relationship between body mass and body length for the two behavioral phenotypes. Lastly, we examined repeated measures of body mass for individuals that alternated between calling and satellite behavior (using two-tailed paired t-tests) to determine if satellites were in significantly poorer condition than callers.

Significant differences are reported for $\alpha < 0.05$ in all analyses. Data were analyzed using Statview Statistical Software (SAS Institute Inc., Cary, North Carolina).

RESULTS

Hormone Levels

Mean steroid hormone levels of callers and satellites were similar for *B. woodhousii* and *B. cognatus* (Fig. 1). There were no significant differences in DHT ($F_{1,110} = 0.41$, $p = 0.5$) or T levels ($F_{1,110} = 2.10$, $p = 0.1$) for satellite and calling *B. woodhousii*, but B levels were significantly higher for callers ($F_{1,110} = 3.90$, $p = 0.04$; Fig. 1A). There were also no significant differences in circulating DHT ($F_{1,43} = 0.05$, $p = 0.8$) or T levels ($F_{1,43} = 0.001$, $p = 0.9$) for satellite and calling *B. cognatus*. However, B concentrations were again significantly higher for callers ($F_{1,43} = 11.32$, $p = 0.001$; Fig 1B). Although the intra- and inter-assay variation was greater than 20% for DHT, this variation does not mask potential differences in DHT levels between the tactics because: 1) individual blood samples were randomized within each assay so that callers and satellites were represented in each assay, 2) T and DHT levels within an individual are tightly correlated in the current data set (data not shown), and 3) standard errors of the mean for DHT and T were similar or of the same magnitude between callers and satellites.

To compare our results with those reported for *R. catesbeiana* (Mendonça et al., 1985) we examined total androgen levels (T + DHT) for calling and satellite

B. woodhousii and *B. cognatus*. Results remained similar in that there were no significant differences in total androgen concentrations for callers versus satellites in *B. woodhousii* ($F_{1,110}$ = 0.98, p = 0.3) or *B. cognatus* ($F_{1,43}$ = 0.02, p = 0.8).

To further investigate the potential for a reciprocal interaction between B and circulating androgens, we examined linear regressions for B versus T + DHT for calling *B. woodhousii* and *B. cognatus*. There was a weak positive relationship between circulating B and total androgens for *B. woodhousii* ($r^2_{1,81}$ = 0.04, p = 0.06) and *B. cognatus* ($r^2_{1,25}$ = 0.1, p = 0.02; Fig. 2).

<u>Body Condition</u>

Males practicing a satellite tactic were significantly smaller than callers for *B. woodhousii* ($F_{1,148}$ = 6.9, p = 0.009; mean ± SE: satellites: 74 ± 1.1 mm, callers: 78 ± 0.8 mm) and *B. cognatus* ($F_{1,57}$ = 14.5, p = 0.0003; satellites: 81 ± 1.5 mm, callers: 87 ± 0.8 mm). Sample sizes differ from those reported for hormone analyses because statistical analyses of snout-ischial length (SIL) included individuals that were observed and measured but not sampled for hormone levels. Results were similar when analyses included only individuals from which blood was collected.

Differences in SIL for the two behavioral phenotypes precluded the use of condition indices (i.e., calculating individual scores based on the proportion of body mass to body length) because ratios do not control for SIL (Tomkins and Simmons, 2002). We therefore followed the statistical procedures outlined by Tomkins and Simmons (2002) to determine if satellites differed from callers in relative body condition. ANCOVA was initially performed (on log-transformed data) with body mass as the dependent variable, behavior (satellite versus caller) as the factor, and SIL as the covariate. Analysis for *B. woodhousii* revealed a significant interaction between mass and SIL ($F_{1,121} = 11.07$, $p = 0.001$; Fig. 3). There was no significant interaction between mass and SIL for satellite versus calling *B. cognatus* ($F_{1,41} = 0.56$, $p = 0.4$) and satellite males were not in significantly poorer condition than callers ($F_{1,41} = 0.50$, $p = 0.4$).

Because heterogeneity of slopes violates the assumptions of ANCOVA (Sokal and Rohlf, 1981), log body mass for satellite and calling *B. woodhousii* was adjusted to the common mean log SIL (1.88 mm) using the equation calculated from the pooled slope (see Tomkins and Simmons, 2002). ANOVA was then used to determine if satellites and callers differed significantly in body mass corrected to the mean SIL. Satellite *B. woodhousii* were in significantly poorer condition than callers ($F_{1,123} = 10.41$, $p = 0.001$).

To visually examine the body mass distribution of the two behavioral phenotypes, residual body mass values obtained from a pooled regression of satellite and calling

B. woodhousii and *B. cognatus* were plotted against SIL. The majority of *B. woodhousii* satellites (76%) fell below the zero y-axis value whereas callers were approximately equally distributed above (52%) and below (48%) the line (Fig. 4A), indicating that satellites were generally in poorer condition than callers. In contrast, only 57% of satellite *B. cognatus* fell below the zero y-axis whereas 46% of callers were distributed above the line (Fig 4B).

We further examined the relationship between relative condition and behavior by comparing repeated measures of body mass from individuals that were observed to alternate between calling and satellite behavior. Repeated measures were taken from only a few individuals because handling and measuring procedures potentially have adverse effects on subsequent hormone levels and behavior (Moore et al., 1991). In the present study, we were interested in determining natural changes in behavioral expression and, therefore, we attempted to minimize handling and did not collect multiple blood samples from individual toads. Because of the small number of individuals repeatedly sampled (n = 4 for *B. cognatus* and n = 14 for *B. woodhousii*), we combined the data for both species to examine differences in body mass for callers versus satellites. A paired t-test (two-tailed) indicated that individuals were significantly heavier when calling compared to when they were exhibiting satellite behavior ($t_{1,17}$ = 3.91; p = 0.001; Fig. 5). The duration between repeated measures of body mass associated with transitions in behavior ranged from 47-195 hrs for *B. woodhousii* (mean ± SE = 120 ± 14 hrs) and 23-72 hrs for *B. cognatus* (36 ± 12 hrs). Individuals practicing a satellite tactic were, on average, 4.0 g lighter than when they were calling. Based on the mean body mass for each species (*B. cognatus* = 67.0 g; *B. woodhousii* = 49.0 g), this difference represents a 6% to 8% reduction in body mass.

DISCUSSION

Circulating levels of androgens (DHT and T) did not differ for calling and satellite *Bufo woodhousii* or *B. cognatus* despite significantly higher circulating B levels for calling males. Further investigation revealed no evidence of a significant interaction between B and androgens in either species. Given that individual *B. woodhousii* and *B. cognatus* were observed alternating between behaviors, a significant decrease in relative body condition while exhibiting satellite tactics suggests that high B levels in calling males are associated with depletion of energetic reserves during vocal production. Differences in body condition are not likely to be the result of differential hydration states for the two behavioral categories because all individuals remained partially submerged while calling or exhibiting satellite behavior. Our results suggest that satellites are physiologically constrained from sustained calling activity and must forage prior to initiating subsequent calling bouts (Marler and Ryan, 1996). Sullivan (1982b) reported that tactic expression in *B. cognatus* is also influenced by social context, because satellite males typically adopted calling behavior following removal of callers from the chorus. In Sullivan's (1982b) investigation, focal observations were terminated when satellites were observed to call (B.K. Sullivan, pers. comm.). Using similar procedures, we have found that satellites sometimes adopt vocal behavior after removal of the associated host as reported by Sullivan (1982b), but only for a very brief period of time. Individuals subsequently resume satellite behavior after ephemeral bouts of calling (CJL, pers. obs.).

Although satellites had lower B levels than callers in both toad species, B concentrations showed a 1.5-fold difference for satellite and calling *B. woodhousii* but a 3-fold difference for satellite and calling *B. cognatus*. These differences may be due to species-specific differences in B sensitivities and/or production but we also suspect that these results are attributable, in part, to striking behavioral differences between the two species. Satellite *B. cognatus* parasitize a single vocalizing individual by remaining stationary and in close proximity of their host often for prolonged periods of time (i.e., in excess of 2 hours; CJL, pers. obs.). In contrast, satellite *B. woodhousii* actively patrol the periphery of chorusing males and persistently engage in amplexus with other males in an attempt to locate incoming females (non-discriminating male–male amplexus is common in *Bufo*; Leary, 2001a,b). This satellite tactic in *B. woodhousii* was also reported by Sullivan (1989) and is similar to that reported by Forester and Thompson (1998) for *B. americanus* (referred to as "gauntlet behavior"). Satellite *B. woodhousii* in our study population, however, are not terrestrial (as reported for *B. americanus*) but are restricted to deeper water surrounding calling males located in shallow water. Hence, non-calling satellite *B. woodhousii* may exhibit higher B levels relative to callers than do *B. cognatus* satellites because satellite *B. woodhousii* continue to exhibit energetically demanding activities (i.e., swimming/frequent amplexus), whereas satellite *B. cognatus* characteristically remain inactive.

Territorial and aggressive interactions have been shown to elevate circulating B and suppress androgen production in numerous vertebrates (Greenberg and Crews, 1990; Greenberg and Wingfield, 1987; Knapp and Moore, 1995, 1996). However, Emerson and Hess (2001) have questioned whether a reciprocal relationship exists between B and androgens for territorial *R. catesbeiana* (as reported by Mendonça et al., 1985). Emerson and Hess (2001) pointed out that satellite individuals in many species have a larger testes to body-size ratio which may provide an alternative explanation for observed elevations in circulating androgens in satellite *R. catesbeiana*. Our data do not reveal a reciprocal relationship for these hormones in non-territorial *B. woodhousii* and *B. cognatus,* but we do not have sufficient data to adequately address Emerson and Hess' (2001) hypothesis. Differences in testes size may still exist in satellite and calling *B. woodhousii* and *B. cognatus* regardless of the fact that behavioral phenotypes are not "fixed" in these species because for the majority of anurans studied thus far (including *Bufo* and *Rana*) satellites are significantly smaller and presumably younger. Therefore, differences in gonosomatic "indices" may be a function of allometric growth rates. Currently, comparative analyses are not sufficient to determine whether general differences in hormonal patterns exist for territorial and non-territorial anuran species. Behavioral differences among *R. catesbeiana*, *B. woodhousii,* and *B. cognatus* may thus contribute to interspecific differences in steroid hormone profiles for satellites versus callers.

Ecological factors may also play a role in interspecific differences in steroid hormone profiles among anurans. For instance, in the population studied by Mendonça et al. (1985), *R. catesbeiana* bred over a prolonged period (several months) whereas *B. woodhousii* and *B. cognatus* are explosive breeders, often completing the breeding season in less than one week (Krupa, 1989; Sullivan, 1982b, 1983, 1989; Wells, 1977; CJL, pers. obs.). Explosively breeding arctic bird species modulate adrenocortical responses to stressors (e.g., Wingfield et al., 1995; 1998) by altering sensitivity to elevated B (e.g., by varying B binding protein and/or receptor levels, reviewed by Breuner and Orchinik, 2002). Physiological and/or behavioral regulation of B (i.e., alternating between vocal and non-vocal behavior) would potentially be advantageous in anuran species with a narrow window of opportunity for reproduction. For example, assuming that elevated androgens are responsible for the production and/or maintenance of sexual behavior (Harvey et al., 1997), a reciprocal interaction between B and androgens in an explosive breeding species would potentially remove individuals from the pool of conspecifics engaged in breeding activity for a significant portion of the reproductive period. In addition, elevated androgens are necessary to initiate spermatogenesis (Harvey et al., 1997). Hence, strong selective pressures may maintain high androgens in explosive breeding species in order to maximize individual reproductive potential.

Regardless of behavioral and/or ecological differences and potential physiological mechanisms that may modulate stress responses, our results suggest that decision rules governing the expression of satellite tactics in *B. woodhousii* and *B. cognatus* are independent of androgens. Although other studies have examined the relationship between steroid hormone levels and calling/non-calling behavior in anurans (reviewed by Burmeister et al., 2001), comparisons may not be appropriate because non-calling behavior is set within very different social and ecological contexts. For instance, Townsend and Moger (1987) examined non-calling male *Eleutherodactylus coqui* exhibiting parental care of terrestrial eggs, Marler and Ryan (1996) examined non-calling male *Physalaemus pustulosus* foraging away from breeding ponds, and Harvey et al. (1997) studied non-calling “prebreeding” and “postbreeding” male *Scaphiopus couchii*. Mendonça et al. (1985) and the present study focus on non-calling satellite males that commonly intercept females for mating. One commonality among the 3 anuran species for which there is information specifically addressing alternative non-calling satellite tactics is that callers have elevated levels of B. It may be that glucocorticoids mediate the expression of alternative mating tactics in anurans without necessarily having a negative effect on androgen production. One such mechanism could involve direct B-mediated regulation of arginine vasotocin (AVT).

AVT is produced in sexually dimorphic vocal control centers of the anuran brain and induces calling behavior when administered to males of various frog and toad species (Wilczynski and Chu, 2001), including *B. cognatus* (Propper and Dixon, 1997). A role of AVT in mediating reproductive behaviors has also been demonstrated in other vertebrates including salamanders (Moore et al., 2000), fishes (Bastian et al., 2001; Semsar et al., 2001), mammals (vasopressin is the mammalian homologue, Bester-Meredith and Marler, 2001) and birds (Voorhuis et al., 1991). AVT production, in turn, can be regulated by androgens (Boyd, 1994a,b). When this process is incorporated into the Energetics–Hormone Vocalization Model proposed by Emerson (2001), it suggests that cyclic fluctuations in androgen levels are accompanied by fluctuations in AVT production. Such a mechanism may explain cyclic patterns of behavioral expression in bullfrogs but does not appear to be occurring in *B. woodhousii* or *B. cognatus*. Therefore, we need to look elsewhere for a potential mechanism mediating behavioral tactics in these species.

Corticosterone acts directly on AVT neurons in the rough-skinned newt, *Taricha granulosa*, via a non-genomic mechanism (i.e., does not require gene transcription, Moore and Evans, 1999; Orchinik et al. 1991). In this species, AVT also mediates courtship behavior (i.e., clasping responses associated with amplexus; Moore et al., 2000; Rose et al., 1995), which can be directly and rapidly inhibited by exogenous application of corticosterone (Moore and Miller, 1984; Rose et al., 1993; 1998). If the results obtained in *T. granulosa* are applicable to anurans, then direct effects of B on AVT neurons may regulate cyclic patterns of calling and non-calling activity. Burmeister et al. (2001) addressed this possibility in green treefrogs (*Hyla cinerea*), but the results were equivocal. In túngara frogs, B implants suppressed calling activity, but the treatment caused a significant decrease in circulating androgens (Marler and Ryan, 1996). Hence, it was unclear if effects resulted from a decrease in androgens or were a direct result of B.

We propose a model (Fig. 6) that incorporates AVT to explain cyclic patterns of calling and satellite behavior in *B. woodhousii* and *B. cognatus*. This new model is based on our data for relative body condition and steroid hormone profiles for these species, models interrelating energetics and corticosterone action (i.e., concepts of the "Emergency Life History Stage" theory) proposed by Wingfield and Ramenofsky (1999), and the Energetics–Hormone Vocalization Model proposed by Emerson (2001). Our model differs from previous models in that it proposes a B-mediated response that is independent of the hypothalamic–pituitary–gonadal axis (i.e., there is no reciprocal interaction between B and androgens). The model proposes that fluctuations in androgen concentrations coincide with AVT synthesis/release (Fig. 6). Periodic elevations in androgens are based on findings reported for other anuran species (reviewed by Emerson and Hess, 2001), and the association between androgens and AVT is based on Boyd (1994a,b). Corticosterone concentration, in turn, parallels calling duration (Emerson and Hess, 2001), but prolonged calling (over several consecutive nights) potentially elevates circulating B (depending on condition and prey availability) and initiates interactions between B and AVT-producing neurons when threshold levels are reached (Fig. 6). Our model does not incorporate call effort, which is expected to coincide with androgen and B levels (Emerson, 2001), nor does it consider the entire breeding season (i.e., androgens are expected to diminish towards the end of the breeding season, Itoh et al., 1990).

To date, only one study in anurans bears on our model and these data may be consistent with a non-genomic effect of B on AVT neurons. Satellite male cricket frogs (*Acris crepitans*) had higher concentrations of forebrain AVT than callers, suggesting that

AVT synthesis is not inhibited in satellites (Marler et al. 1999). However, potential depletion of AVT reserves during vocal production complicates determining whether or not AVT synthesis is inhibited, and steroid hormone profiles for callers and satellites are not available for this species.

Further investigations and comparative studies are necessary to elucidate the proximate mechanisms regulating the expression of non-calling satellite tactics in anurans. Future investigations should establish natural steroid hormone levels for satellites and callers given that numerous ecological factors (i.e., duration of breeding season) and behavioral characteristics (i.e., aggression) vary from species to species and potentially contribute to interspecific variation in physiology. The differences in steroid hormone profiles for satellites and callers in the species studied thus far suggest two different pathways that may regulate expression of alternative mating tactics in anurans: a direct B–AVT mediated response versus a B–androgen–AVT mediated response. We are currently working to distinguish between these two potential mechanisms in *B. woodhousii* and *B. cognatus*.

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FIGURE LEGENDS

Figure 1. Mean (± SE) steroid hormone levels for satellite and calling male (A) *Bufo woodhousii* and (B) *B. cognatus*. Dihydrotestosterone (DHT) and testosterone (T) were not significantly different for the two behavioral categories, but corticosterone (B) was significantly higher in callers (indicated with an asterisk).

Figure 2. Linear regressions showing the relationship between corticosterone and total androgens (testosterone, T + dihydrotestosterone, DHT) in (A) *Bufo woodhousii* and (B) *Bufo cognatus.*

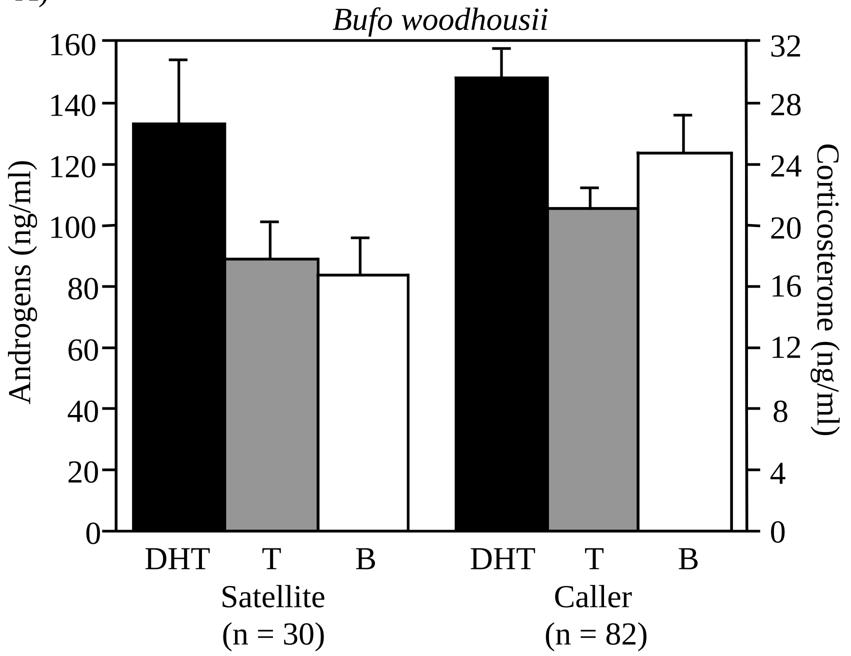
Figure 3. Relationship between log snout-ischial length and log body mass for satellite (solid circles) and calling (open circles) *Bufo woodhousii*. There was a significant interaction between the two variables across behavioral phenotypes (see text).

Figure 4. Regression of residual values of log body mass plotted against snout-ischial length for satellite and calling (A) *Bufo woodhousii* and (B) *B. cognatus*. The majority of satellites fell below the zero y-axis value for both species (see text).

Figure 5. Changes in body mass associated with transitions in behavior for individual *Bufo woodhousii* and *B. cognatus*. Data on the left side of the figure are from individuals first observed calling and subsequently observed exhibiting satellite behavior; data on the right side are from individuals first observed exhibiting satellite behavior and then subsequently observed calling. Solid circles represent an individual *B. woodhousii* that was observed alternating between behaviors on two occasions.

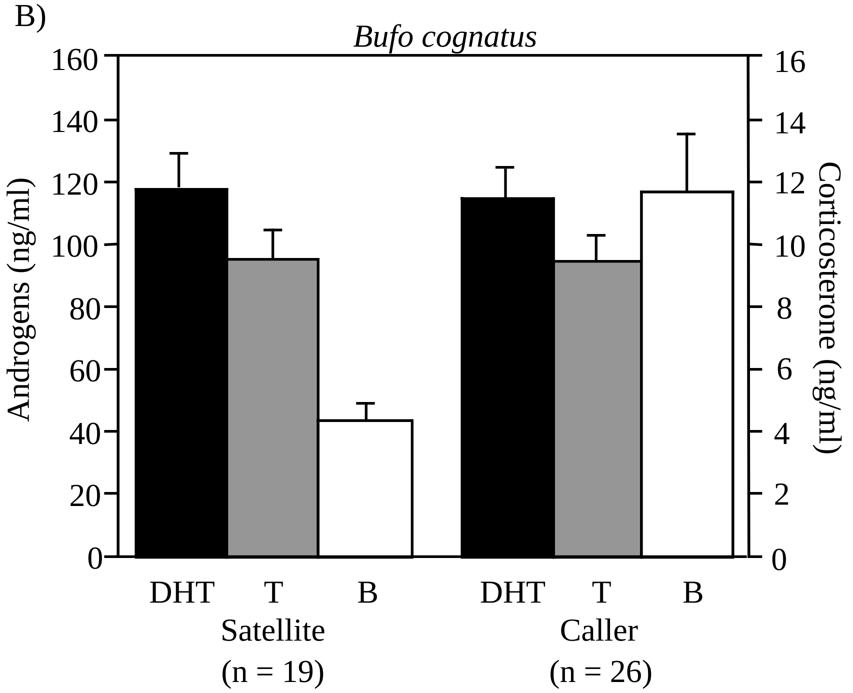
Figure 6. Proposed mechanism controlling alternation between calling and satellite behavior in *Bufo woodhousii* and *B. cognatus*. The model proposes a reciprocal interaction between corticosterone (B) and arginine vasotocin (AVT) secretion. Elevated B levels are driven by depletion of energetic reserves over time (between successive calling bouts) and probably fluctuate during inactive daytime periods and active night time periods. The slopes for B concentration and energetic reserves is dependent upon condition and prey availability. Androgens exhibit temporal fluctuations that result in fluctuations in AVT synthesis, but androgens do not exhibit a reciprocal relationship with B.

A)



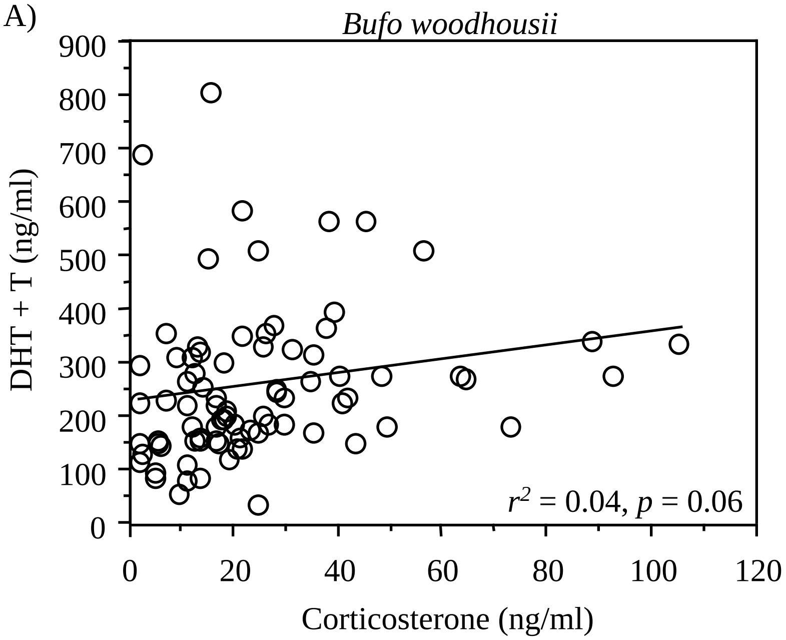
Bufo woodhousii
Androgens (ng/ml)
0
20
40
60
80
100
120
140
160
Corticosterone (ng/ml)
0
4
8
12
16
20
24
28
32
DHT
T
B
Satellite
(n = 30)
DHT
T
B
Caller
(n = 82)

B)



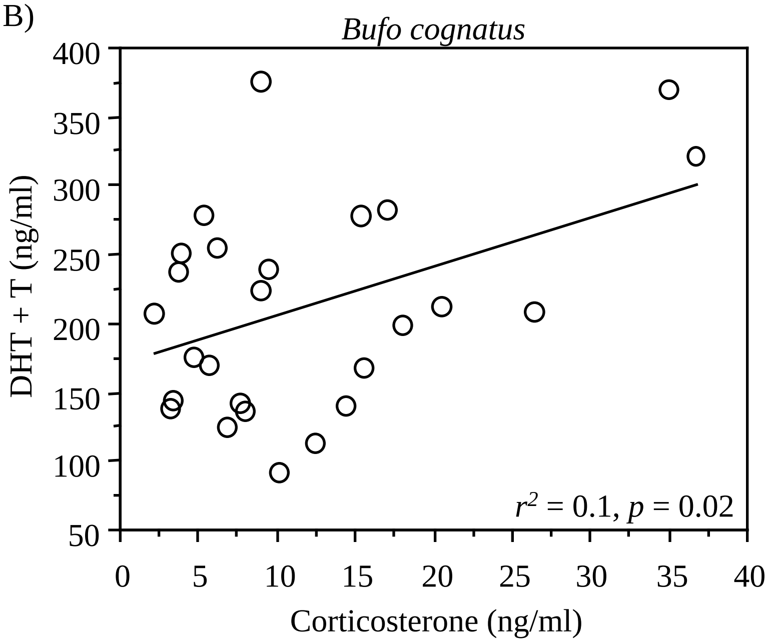
Bufo cognatus
Androgens (ng/ml)
0
20
40
60
80
100
120
140
160
Corticosterone (ng/ml)
0
2
4
6
8
10
12
14
16
DHT
T
B
Satellite
(n = 19)
DHT
T
B
Caller
(n = 26)

A)

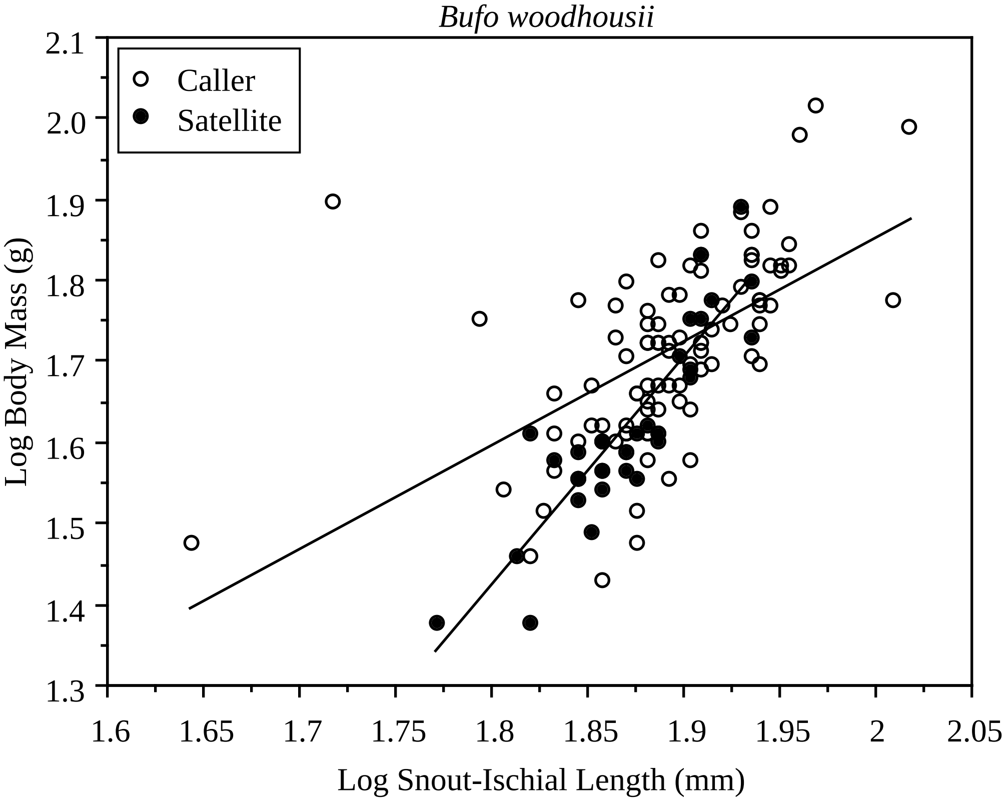


Bufo woodhousii
DHT + T (ng/ml)
0
100
200
300
400
500
600
700
800
900
0
20
40
60
80
100
120
Corticosterone (ng/ml)
r^2 = 0.04, *p* = 0.06

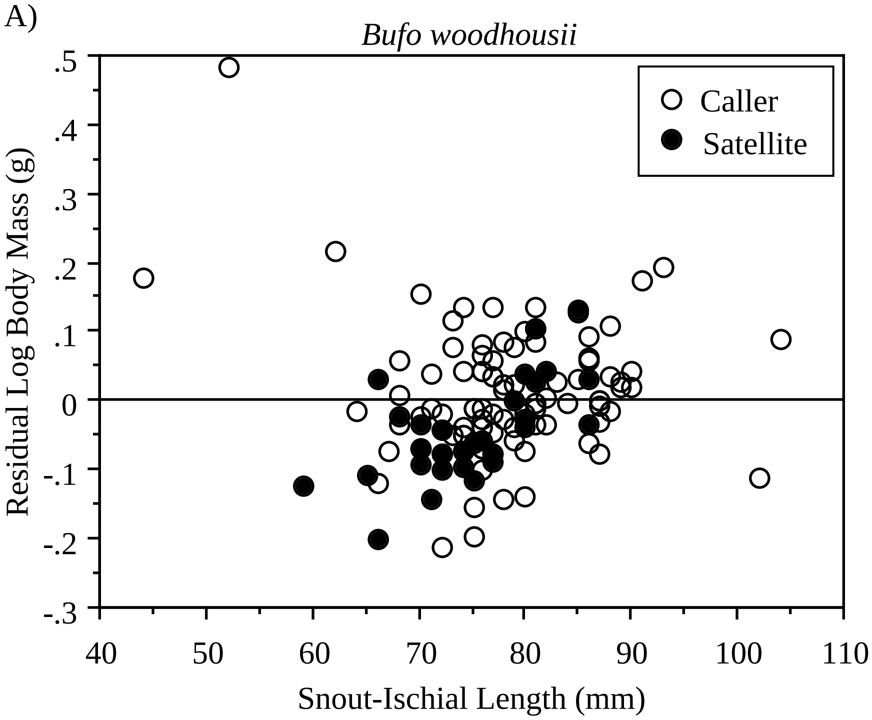
B)



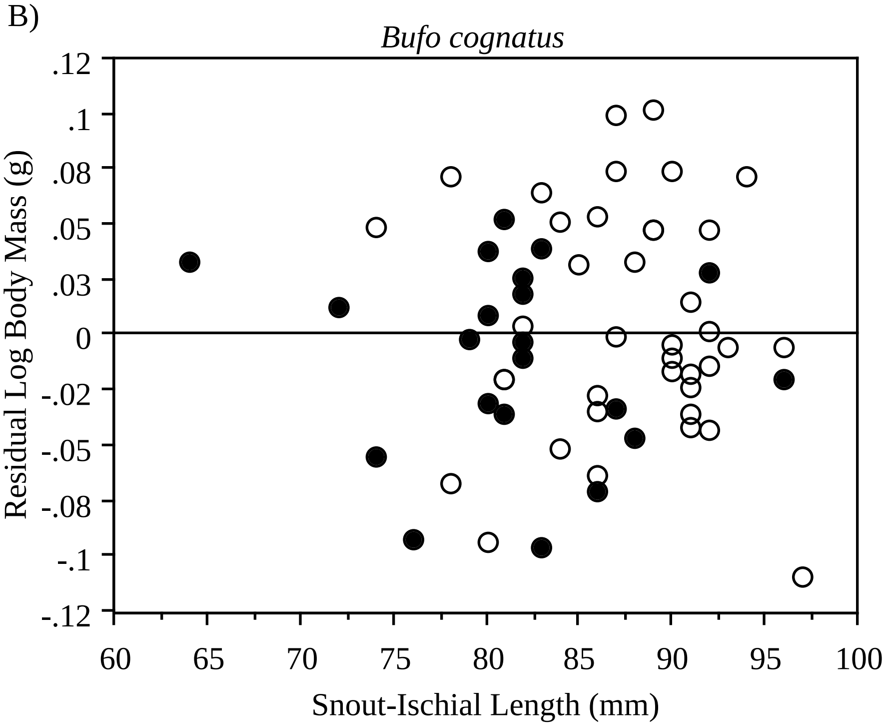
Bufo cognatus
DHT + T (ng/ml)
50
100
150
200
250
300
350
400
0
5
10
15
20
25
30
35
40
Corticosterone (ng/ml)
r^2 = 0.1, *p* = 0.02

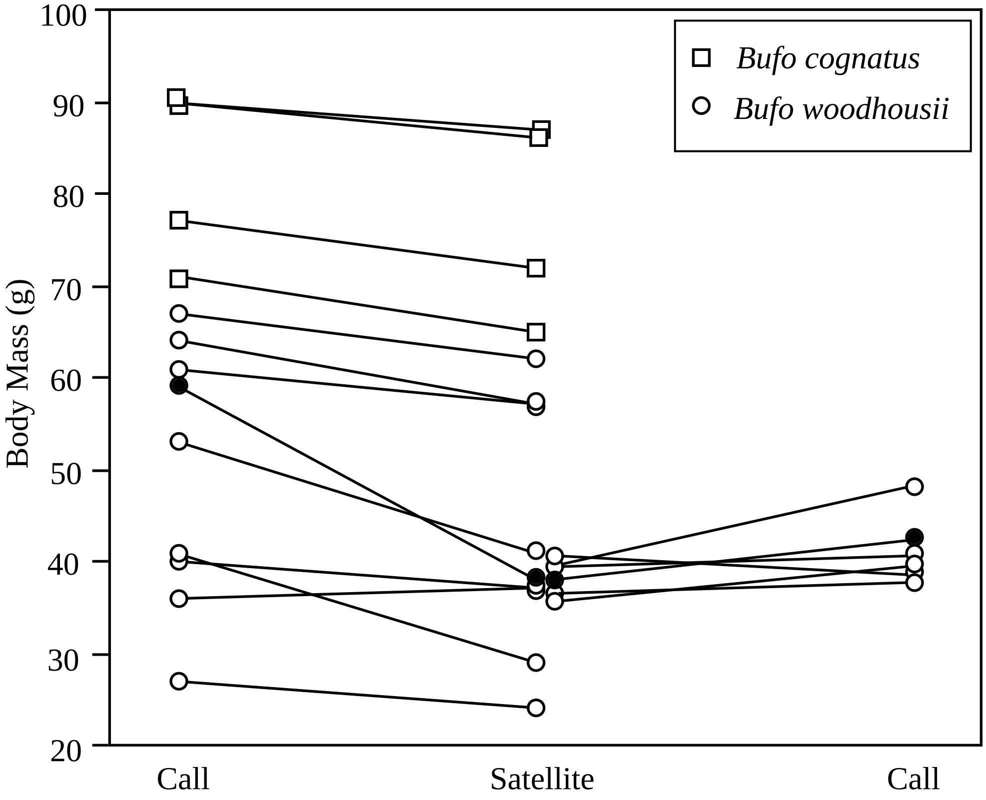

Bufo woodhousii
Caller
Satellite
Log Body Mass (g)
Log Snout-Ischial Length (mm)
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.6
1.65
1.7
1.75
1.8
1.85
1.9
1.95
2
2.05

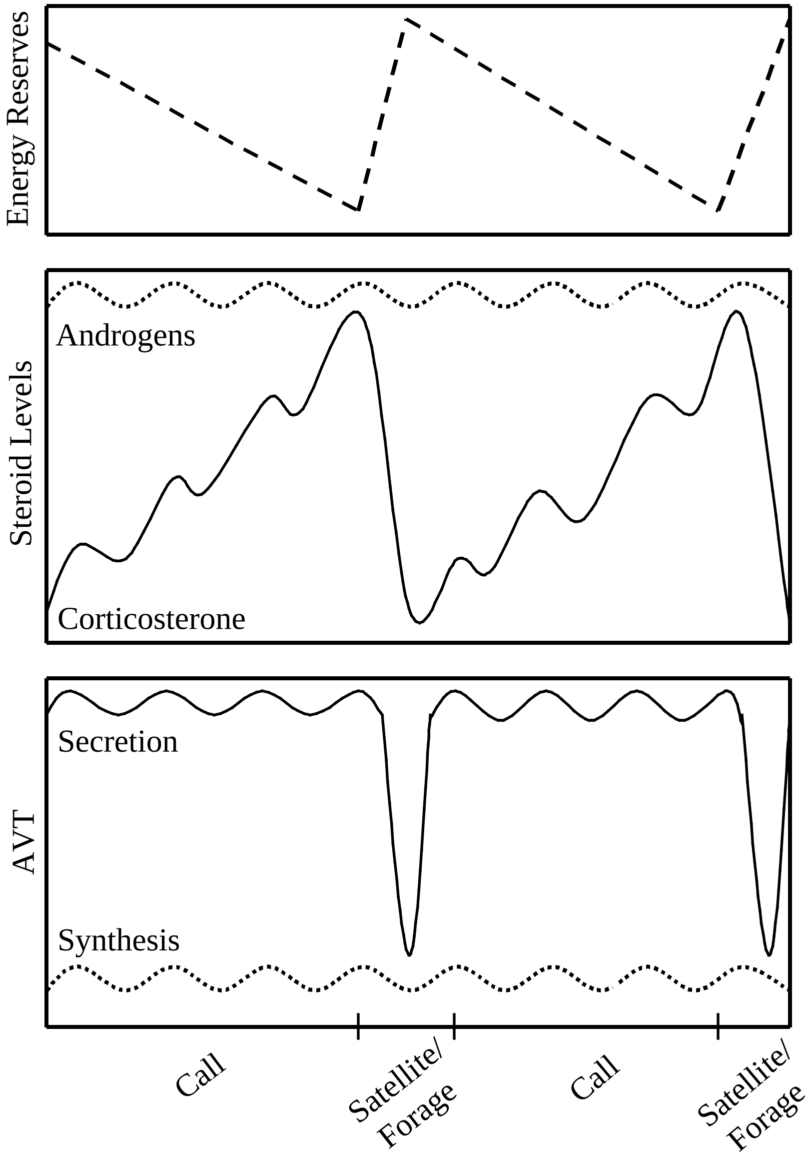
A)


Bufo woodhousii
○ Caller
● Satellite
Residual Log Body Mass (g)
.5
.4
.3
.2
.1
0
-.1
-.2
-.3
40
50
60
70
80
90
100
110
Snout-Ischial Length (mm)

B)


Bufo cognatus
Residual Log Body Mass (g)
.12
.1
.08
.05
.03
0
-.02
-.05
-.08
-.1
-.12
60
65
70
75
80
85
90
95
100
Snout-Ischial Length (mm)


Body Mass (g)
100
90
80
70
60
50
40
30
20
Call
Satellite
Call
Bufo cognatus
Bufo woodhousii


Energy Reserves
Steroid Levels
Androgens
Corticosterone
AVT
Secretion
Synthesis
Call
Satellite/ Forage
Call
Satellite/ Forage

Chapter II

Elevated Corticosterone Levels Elicit Alternative Non-Calling Mating Tactics in Male Toads

ABSTRACT--. Competition among males for a limited number of females may result in the expression of condition-dependent alternative mating tactics. In such cases, decision rules mediating mating tactic expression are likely to be influenced by extrinsic as well as intrinsic conditions. For example, experimental studies with anuran amphibians (frogs and toads) indicate that changes in the social-acoustic environment alter the probability that an individual adopts a calling or non-calling "satellite" mating tactic. However, there is considerable variation in the behavioral responses of individuals in such studies, suggesting that physiological differences among individuals may play an important role in tactic expression. For instance, recent models predict that natural elevations in adrenal glucocorticoids during vocal production may alter androgen production and/or neural activity to mediate transitions in reproductive tactic expression in anuran amphibians. Using corticosteroid injections, we show that elevations in circulating corticosterone levels significantly increase the probability that free-ranging male Great Plains toads (*Bufo cognatus*) and Woodhouse's toads (*B. woodhousii*) adopt non-calling satellite tactics. Corticosterone-induced behavioral transitions occurred rapidly (<1 hour) and independently of fluctuations in circulating androgen levels, suggesting a direct effect of glucocorticoids on arginine vasotocin-producing neurons in the vocal motor pathway. Our findings have important implications regarding behavioral studies that alter the

social-acoustic environment to examine its influence on tactic expression, and dynamic game models wherein tactic expression is rooted in the energetic state of the organism.

Introduction

Condition-dependent variation in male reproductive behavior often involves complex interactions between the organism's environment and internal physiological state (Andersson, 1994; Shuster and Wade, 2003; West-Eberhard, 2003). Hence, an understanding of individual variation in sexual behavior often requires analysis of the environmental factors that influence behavioral expression as well as the physiological parameters that alter the probability of a given behavioral response under some set of environmental conditions (Crews and Moore, 1986; Halliday, 1987, 1992; Mayr, 1982; Andersson, 1994; Drickamer, 1998; Drickamer and Gillie, 1998; Sinervo and Svensson, 1998). Anuran amphibians (frogs and toads) are one taxonomic group in which a considerable number of investigations have focused on altering the social environment to examine its influence on the expression of alternative mating behaviors. However, less is known regarding the role that the animal's physiological state plays in the adoption of such tactics. Here, we focus on the effects of elevated circulating glucocorticoid levels on male mating tactic expression in chorusing Great Plains toads (*Bufo cognatus*) and Woodhouse's toads (*B. woodhousii*). Elevations in adrenal glucocorticoid levels are associated with reproductive activity for most anurans examined thus far, as well as numerous other vertebrate species (Romero, 2002; Love et al., 2004), and are a key component of recent models regarding reproductive tactic expression for this group (Emerson, 2001; Leary et al., 2004).

In many anuran species, males exhibit alternative “satellite” tactics wherein non-calling individuals reside in close proximity to calling conspecific males and attempt to intercept incoming females attracted to the vocalizations of the calling host (Waltz, 1982; Halliday and Tejedo, 1995; Gerhardt and Huber, 2002). Individuals can alternate between calling and non-calling satellite tactics within and between nights of chorus activity (reviewed by Halliday and Tejedo, 1995), suggesting that satellite behavior in this clade is a condition-dependent mating tactic (sensu Gross, 1986).

One common approach to studying satellite behavior in anurans has been to alter the social-acoustic environment to examine its influence on behavioral expression. The working hypothesis of these studies proposes that information is encoded within the vocalizations of conspecific males (i.e., relative attractiveness and/or social status) and that receivers use this information in mating tactic decisions. For example, Arak (1988) predicted that males should adopt satellite behavior when the mean sound pressure intensity (a measure of relative amplitude) of chorusing conspecifics is at least twice as great as the subject’s call intensity because the probability of obtaining a mate would be greater if an “unattractive” male adopted a satellite tactic. Similarly, Waltz (1982) applied the “law of diminishing returns” to predict patterns of satellite behavior wherein mate quality and attractiveness drive behavioral expression. Currently, such models are partially supported by vocal playback experiments and satellite/caller manipulations. For example, in playback experiments of varying amplitude, 56% of 16 calling male natterjack toads (*Bufo calamita)* adopted satellite behavior when presented with a high amplitude conspecific vocal stimulus whereas 25% of 16 calling males became satellites when subjected to a low amplitude stimulus (Arak, 1988). Conversely, satellite *B. calamita* adopted calling behavior in only 20% of 10 satellite-host manipulations where the caller was removed from the vicinity of the satellite male (Arak, 1988). Similarly, in gray treefrogs (*Hyla versicolor),* Fellers (1979) reported that 28% of 18 calling males adopted satellite tactics when a calling male was placed nearby. In the green treefrog (*Hyla cinerea*), only 18% of 17 satellite males exhibited calling behavior following cessation of vocal production by the host (Perrill et al., 1978). However, in playback experiments, 63% of 8 calling male *H. cinerea* adopted satellite tactics following exposure to conspecific advertisement calls (Perrill et al., 1982) whereas only 13% of 24 male cricket frogs (*Acris cepitans)* adopted satellite tactics during similar experimental procedures (Perrill and Magier, 1988). Comparable results were obtained for *Uropeila rugosa* (Robertson, 1986a,b)*, Acris crepitans* (Wagner, 1992; Burmeister et al., 1999), *Philautus variabilis* (Kadadevaru and Kanamadi, 2001), and *Rana rugosa* (Park and Cheong, 2002). The responses of individuals in such studies vary considerably (across species as well as within species), suggesting that differences in physiology may play an important role in observed differences in behavioral responses among individuals exposed to similar social and/or environmental conditions.

Reproductive behavior and vocal communication in anurans are known to controlled by two chemical systems, including gonadal steroids and the neuropeptide arginine vasotocin (Marler et al., 1999; Wilczynski and Chu, 2001). Currently, few studies have documented natural variation in these systems among individual anurans practicing alternative mating tactics (i.e., Mendonça et al., 1985; Marler et al., 1999; Leary et al., 2004). For example, Mendonça et al. (1985) found that calling male bullfrogs (*Rana catesbeiana*) had significantly lower circulating levels of androgens and significantly higher levels of circulating corticosterone than satellite males. An inverse interaction between the two steroids was predicted to account for transitions between calling and satellite behavior in this species.

The potential effects of corticosteroid administration on reproductive tactic expression have not been examined in bullfrogs, but glucocorticoid administration and/or natural elevations in glucocorticoids have been found to be inversely correlated with circulating androgen levels in several other anuran species (see Bambino and Hsueh, 1981; LeBoulenger et al., 1982; Licht et al., 1983; Townsend and Moger, 1987; Marler and Ryan, 1996; Harvey et al., 1997) as well as numerous reptiles, mammals, and birds (e.g., Greenberg and Wingfield, 1987; Greenburg and Crews, 1990; Knapp and Moore, 1995, 1996; Schuett et al., 1996; Wingfield and Ramenofsky, 1999). Such a relationship was combined with concepts of the "Challenge Hypothesis" (Wingfield et al., 1990) by Emerson (2001) to explain transitions between calling and non-calling activity in anuran amphibians. Emerson's (2001) "Energetic-Hormone Vocalization Model" predicted a positive relationship between androgen levels and vocal effort until the energetic demands associated with vocal production increase glucocorticoids to threshold levels that inhibit androgen production. At this stage, a calling male stops vocalizing until circulating glucocorticoids are metabolized and energy reserves are restored. Subsequent to recovery from this stress response, an individual resumes calling behavior and continues this cyclic pattern throughout the breeding period.

Leary et al. (2004) proposed that elevated glucocorticoids may directly alter reproductive tactic expression in explosive-breeding male toads independently of fluctuations in androgen levels by altering activity of AVT-producing neurons in vocal control centers of the brain. Our proposed model stemmed from the lack of evidence for a reciprocal interaction between corticosterone and androgens in satellite and calling Woodhouse's toads (*B. woodhousii*) and Great Plains toads (*B. cognatus*) (Leary et al., 2004; Leary et al., *in review*) and glucocorticoid/arginine vasotocin (AVT) relationships in the rough skinned newt (*Taricha granulosa*). AVT mediates courtship behavior in rough skinned newts and behavioral expression can be rapidly altered (within seconds to minutes) by exogenous application of corticosterone (Moore and Miller, 1984; Rose et al., 1993, 1998). Corticosterone appears to inhibit reproductive behavior in rough skinned newts by altering the secretion of AVT from neurons via a nongenomic mechanism (Moore and Evans, 1999; Orchinik, 1991). A similar mechanism may explain higher levels of AVT-immunoreactivity in vocal control centers of satellite versus calling cricket frogs (*Acris crepitans*) documented by Marler et al. (1999).

Endocrine-related investigations on anuran vocal behavior indicate that glucocorticoids can mediate cyclic patterns of calling and non-calling behavior, a finding that has been supported by hormone manipulation studies (Marler and Ryan, 1996; Burmeister et al., 2001). However, the mode of action for glucocorticoids may differ across species (Leary et al., 2004). For example, based on evidence that androgens are required for AVT synthesis (Boyd, 1994a,b), the disparate steroid hormone profiles in anuran species that exhibit satellite behavior suggest two different pathways that regulate expression of alternative mating tactics: a corticosterone–androgen–AVT pathway (*R. catesbeiana*, Mendonça et al., 1985) versus a direct corticosterone–AVT pathway (*B. woodhousii* and *B. cognatus*, Leary et al., 2004). Leary et al. (2004) proposed that such differences in endocrine regulation of vocal behavior in anurans may be related to differences in the temporal frame of reproductive activity for the species examined. This proposition was based on elements of the “Emergency Life History Stage theory” developed by Wingfield et al. (1998). Wingfield and colleagues found through extensive comparative endocrine studies that explosive-breeding Arctic bird species were less likely to exhibit a reciprocal interaction between corticosterone and androgen production than more prolonged breeding temporal species. A similar pattern may exist for anuran species that exhibit (often extreme) differences in temporal patterns of reproduction (see Wells, 1977 for review of explosive and prolonged breeding anuran species).

Application of the Emergency-Life History Stage theory to anuran amphibians may help to clarify some of the results obtained from hormone manipulation studies that have examined the endocrine factors involved in vocal regulation. For example, Burmeister et al. (2001) administered corticosterone to calling male green treefrogs (*Hyla cinerea*) and found equivocal evidence for a direct effect of corticosterone on calling behavior. In this investigation, calling individuals that received corticosterone treatment had lower levels of circulating androgen levels than saline-administered controls, suggesting a reciprocal relationship between corticosterone and androgens levels. In addition, many saline-administered individuals stopped vocalizing (Burmeister et al., 2001). Hence, the authors were unable to determine if individuals that stopped vocalizing subsequent to corticosterone treatment were directly affected by an increase in circulating corticosterone levels, a decrease in circulating androgen levels, or procedures associated with the experiment (i.e., handling/placement in enclosures). The authors also indicated that natural steroid hormone levels for the two behavioral phenotypes have not been ascertained for this species. Hence, it is not known whether physiological or pharmacological doses of corticosterone were administered to calling males or whether the natural pathway potentially mediating transitions between calling and non-calling behavior in this prolonged-breeding species is via a corticosterone–androgen–AVT pathway or a direct corticosterone-AVT pathway.

We have documented natural steroid hormone levels for calling and satellite *B. woodhousii* and *B. cognatus* and we have found no evidence that a reciprocal relationship between circulating corticosterone and androgen levels account for transitions between mating tactics in these species (Leary et al., 2004). Furthermore, there is no evidence that differential gonadal investment among the behavioral phenotypes masks a reciprocal relationship between androgen and corticosterone levels (Leary et al., *in review*). In the present study, we examine tactic expression of chorusing male *B. cognatus* and *B. woodhousii* subsequent to corticosterone and saline treatment. We show that elevations in circulating corticosterone levels increase the probability that free-ranging males adopt non-calling satellite tactics under similar environmental and social circumstances. In addition, we show that behavioral transitions occur rapidly (< 1hr) and independently of fluctuations in circulating androgen levels. Our results are consistent with our hypothesis that corticosterone acts by altering neural activity in vocal control centers of the brain to regulate tactic expression in these species. We relate our findings to behavioral studies that alter the social-acoustic environment to examine its influence on behavioral expression and satellite-caller dynamic game models that are rooted in the energetic state of the organism.

Methods

General Information

This study used calling male *B. cognatus* and *B. woodhousii* in natural choruses in central Oklahoma (Cleveland, Grady, and Canadian counties). Male *B. cognatus* data come from three choruses comprised of 13-30 individuals and *B. woodhousii* data come from two choruses that consisted of 65-150 individuals. All procedures took place during peak periods of reproductive activity in June 2003 and 2004 following heavy rains. These species are explosive breeders and often complete all annual reproductive activity in one week or less (Sullivan, 1982; Krupa, 1989; Leary et al., 2004).

Treatment and Dosage

Calling male toads were administered saline or corticosterone in saline vehicle. Estimated dosages were based on circulating corticosterone levels and body mass measures for these species ascertained in our previous investigation (Leary et al., 2004). Crystalline corticosterone (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol (60 μg corticosterone/0.1 ml EtOH), evaporated and resuspended in a saline solution vehicle (115m*M* NaCl, 2.5 m*M* $KHCO_3$, 1.0 m*M* $CaCl_2$, see Propper and Dixon, 1997). Hence, toads were administered a dose of approximately 0.81μg corticosterone/g body weight for *B. cognatus* and 0.95μg corticosterone/g body weight for *B. woodhousii* based on the mean body mass measures for *B. cognatus* (73.7g) and *B. woodhousii* (62.9g) used in this study. Controls were administered an equal volume (100μl) of saline vehicle only.

Pre-Administration Procedures

Prior to treatment, focal observations were made on calling males for 10-20 min. All subjects used in this study persistently vocalized during the pre-treatment observational period. Individuals that were observed to alternate between calling and non-calling activity during the observational period were not used in this study. Individuals that persistently called for the focal time period were then captured by hand and rapidly bled at the site of capture by cardiac puncture and immediately marked with a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL). Approximately 100μl of blood was obtained from each individual and the entire procedure (cardiac puncture + marking) never took more than 30 sec. Individuals were then promptly returned to their respective calling site and subsequently observed. If calling behavior did not resume within 1 min the animal was not used in this study. Individuals that resumed calling behavior within 1 min were allowed to vocalize for approximately 5 min before they were approached and given either a saline or corticosterone injection. Treatment did not require additional handling of the subject; individuals were approached while they are calling and injected intraperitoneally from the caudolateral abdomin. All subjects used in this study continued to vocalize immediately (<1 min) following injection or did not cease vocal production during injection procedures. Subjects that did not resume vocal production immediately following treatment (<1 min) were removed from the study. Treatment was done in a paired fashion so that males receiving corticosterone injections were paired with males that received saline injections at approximately the same time (within 2 min).

Post-Administration Procedures

Experimental subjects were observed by field assistants with no prior knowledge of the experimental treatment (i.e., whether toads were administered corticosterone or saline). Focal observations continued for 1 hr or until experimental subjects adopted satellite tactics or after 1 hr. Males that stopped vocalizing subsequent to treatment were required to approach conspecific calling males and remain in close proximity to calling males for at least 5 min in order to be scored as “satellite” males. Satellite behavior in the two species differs in that *B. cognatus* typically associate with a single “host” male whereas satellite *B. woodhousii* typically patrol groups of calling males, but do not associate with a specific host male (Leary et al., 2004). Males that adopted satellite tactics subsequent to treatment were approached and a second blood sample was rapidly collected if satellite behavior persisted for the required 5 min period (maximum duration of satellite behavior allowed prior to obtaining second blood sample was 15 min). The paired saline-injected individual was also bled by cardiac puncture at approximately the same time (within 2 min). Corticosterone-treated males that did not stop vocalizing and adopt satellite tactics were bled a second time after 1 hr, as were paired saline controls. The second post-treatment blood sample was obtained in 30 sec or less and individuals were subsequently weighed, measured from tip of snout to end of ischium, and returned to the chorus.

Although initial blood samples and treatments were performed in a paired fashion, we were unable to meet the procedural criteria for all experimental subjects. Hence, 2 corticosterone-treated individuals for each species were paired with a single saline-injected control. In these cases, the post-treatment blood sample was obtained from the saline-injected control at the time the second corticosterone-administered individual was bled. Therefore, blood samples were not obtained at the same time for corticosterone and saline-injected animals in these two cases, rather the second blood sample was obtained from the saline-injected male within 15 min subsequent to obtaining the second blood sample from one of the corticosterone-treated subjects. In addition, we were unable to obtain a second blood sample from one saline-injected *B. woodhousii*. Hence, pre and post treatment effects on hormone levels were not available for that individual. However, treatment effects on behavior are reported. Hence, we obtained pre and post behavioral data from 18 toads (5 individuals treated with corticosterone and 4 treated with saline for each species) and pre a post hormone levels for 17 toads (5 individuals treated with corticosterone and 4 treated with saline for *B. cognatus*; 5 individuals treated with corticosterone and 3 treated with saline for *B. woodhousii*).

Precautions were taken throughout the experiment to minimize handling times, observer movement, and disturbance to the subjects. Treatment groups were also randomly assigned to assistants that assessed temporal changes in behavior so that one treatment group was not consistently designated to any assistant. Most focal observations were made under ambient light conditions, but occasionally low power LED headlamps were used. All behavioral observations were transcribed from analog tape recordings made continuously throughout the observation period.

Radioimmunoassay and Statistical Procedures

Blood samples were run in two assays to assess pre and post-treatment steroid hormone levels. The first assay included all blood samples obtained in 2003 and the second assay contained of all data obtained in 2004 so that repeated measures (blood samples) obtained from a single individual were always run in the same assay. Radioimmunoassay procedures followed those described by Leary et al. (2004) and were validated in this previous investigation. Treatment effects on pre and post-treatment hormone levels for dihydrotestosterone (DHT), testosterone (T), and corticosterone (B) were analyzed using paired t-tests. Non-parametric behavioral data were analyzed using chi-square contingency tests.

Results

Radioimmunoassay results on pre and post-treatment blood samples verified that B administration significantly increased circulating B levels in *B. cognatus* ($t_5 = -3.3$, $p = 0.02$) and *B. woodhousii* ($t_5 = -5.6$, $p = 0.005$; Fig 1). Saline injection did not have a significant effect on circulating B levels in *B. cognatus* (t_4 = -0.84, *p* = 0.4) or *B woodhousii* (t_3 = 0.14, *p* = 0.9; Fig 1).

Despite significant elevations in circulating B, there was no evidence of a decrease in total androgen levels (DHT + T) for B-treated *B. cognatus* (t_5 = -1.07, *p* = 0.3) or *B. woodhousii* (t_5 = -0.31, *p* = 0.7; Fig 2). There was also no significant difference in total pre and post-injection levels of circulating androgens for saline-injected *B. cognatus* (t_4 = -1.6, *p* = 0.2) or *B. woodhousii* (t_3 = -0.88, *p* = 0.5; Fig 2). Only data for combined androgens (DHT + T) are presented because DHT and T are correlated in these species and the results did not change when analyzed separately. Trends in the mean pre and post-treatment circulating androgen levels were in the opposite direction of that expected from a reciprocal relationship between the two steroids (i.e., androgens tended to increase subsequent to treatment for both species).

Seven of the 10 B-treated calling males adopted satellite tactics for at least 5 minutes within 1 hr after treatment (range of time between treatment and adoption of satellite tactics = 20-52 min; mean = 41 min). All saline-injected controls persistently vocalized throughout the duration of the experiment. A single B-administered *B. woodhousii* continued to call for the 1 hr focal period and was the only case where we were unable to obtain a second blood sample from the paired saline control male. Vocal production by this B-treated individual continued despite an increase in the circulating level of B from 42.8ng/ml pre-treatment to 133.9ng/ml post-treatment, and a decrease in the circulating level of androgens (DHT +T) from 651.7ng/ml pre-treatement to 304.9ng/ml post-treatment. This was the only male where B administration had a negative impact on circulating androgen levels. The two other males that did not exhibit satellite tactics for the required 5 min time interval (1 *B. woodhousii* and 1 *B. cognatus*) exhibited rapid alternations between calling and satellite tactics approximately 20-30 min post injection. However, individuals were not scored as satellite males in these two cases because the satellite “bouts” were ephemeral. In other words, these individuals repeatedly stopped vocalizing and subsequently approached calling males, but not for the required 5 min period. Alternations between calling and satellite behavior in these individuals were associated with a notable increase in locomotor activity (swimming in the vicinity of calling males) when compared to the pre-injection period and activity patterns of control subjects. Because these individuals were not scored as satellite males, the number of individuals that we report to have switched from the calling to satellite tactic is likely to be a conservative estimate.

Because of the small sample sizes and similar results for both species, behavioral data for the two species were combined in chi-square analysis. There was a significantly greater probability that an individual adopted a satellite tactic (7 of 10 individuals) subsequent to B injection when compared to saline-injected controls (0 of 8 individuals; Pearson's $X^2 = 9.16$, $p = 0.002$).

Discussion

Experimental hormone manipulations indicate that elevation in circulating B increases the probability that male *B. cognatus* and *B. woodhousii* exhibit satellite tactics. Behavioral transitions occurred rapidly (< 1 hr) and independently of fluctuations in circulating androgen levels. Our results are consistent with the hypothesis that B acts directly on neural activity in vocal motor pathways to alter behavioral expression in these species (Leary et al., 2004). Importantly, however, B injections in the present study elevated mean circulating levels of B above those we have previously measured for these species. For example, natural circulating B levels of calling males (with significantly higher levels of B than satellite males) ranged from 1.6 -36.1 ng/ml in *B. cognatus* and 2.5-104.8 ng/ml in *B. woodhousii* (Leary et al., 2004). In the current investigation, B administration procedures resulted in circulating B levels ranging from 9.3-199.4 ng/ml for *B. cognatus* and 133-266 ng/ml for *B. woodhousii*. We expect that circulating levels must be higher than those reported in Leary et al. (2004) to initiate satellite behavior because B levels in calling males have presumably not yet met threshold levels to initiate satellite behavior (Leary et al., *in review*). Estimated treatment dosages are difficult to ascertain when the level of circulating B is not known for a particular individual prior to treatment. In the current investigation, 3/10 B-treated toads adopted satellite tactics when B levels were elevated within the natural range previously reported by Leary et al. (2004).

Second, it should be noted that satellite behavior in the two species studied differs in that satellite *B. woodhousii* typically swim along the periphery of clusters of calling males, whereas satellite *B. cognatus* often remain stationary and in close proximity to a host male (Leary et al., 2004). This distinction is important because Crespi and Denver (2004) found that elevations in B increase locomotor activity in western spadefoot toads (*Spea hammondii*). Our results are consistent with stimulatory effects of B on locomotor activity in *B. woodhousii*, rendering it difficult to distinguish between the two behaviors for this species. Nevertheless, satellite behavior in male *B. woodhousii* is associated with increased locomotor activity under natural conditions wherein males attempt to intercept incoming females (Sullivan, 1989; Leary et al., 2004). That elevated B levels also increase foraging behavior in spadefoot toads (Crespi and Denver, 2004) provides evidence that supports the models of Emerson (2001) and Leary et al. (2004) wherein males were predicted to forage subsequent to bouts of high energy expenditure associated with calling activity. We have also observed calling male *B. cognatus* under natural conditions to switch to a satellite tactic for 20-30 min and subsequently leave the chorus, forage, and return to the chorus and resume satellite behavior (CJL and AMG, pers. obs.). Individuals that recently switched tactics had higher levels of circulating B than calling males and satellite males (Leary et al., *in review*).

Whether B acts on AVT-producing neurons to alter neuropeptide secretion remains largely speculative at this point. Such a mechanism, however, seems likely given the available evidence. In particular, there is an extensive literature on endocrine regulation of reproductive behavior in rough skinned newts by Moore and colleagues that indicate a direct inhibitory effect of glucocorticoids on AVT-producing neurons. AVT mediates courtship behavior in rough skinned newts (i.e., clasping responses associated with amplexus; Moore et al., 2000; Rose et al., 1995), which can be directly and rapidly inhibited by exogenous application of B (Moore and Miller, 1984; Rose et al., 1993; 1998). Corticosterone appears to bind to membrane-bound receptors in AVT neurons and alter AVT secretion via a non-genomic mechanism (Moore and Evans, 1999; Orchinik et al., 1991; Evans et al., 2000). The mechanisms underlying reproductive behavior in newts may be applicable to some anuran amphibians (Leary et al. 2004) and the time scale of behavioral transitions in the current investigation lend further support for parallel mechanistic relationships in newts and toads.

As with the motor pathways regulating amplexus behavior in newts (see above citations), vocal motor pathways in anurans are composed largely of AVT-producing neurons (Kelley and Brenowitz, 1992; Boyd, 1997; Boyd and Moore, 1992; Boyd et al., 1992) and administration of AVT consistently elicits calling behavior in numerous frog and toad species (Boyd, 1994b; Marler et al., 1999; Klomberg and Marler 2000; Burmeister et al., 2001; Wilczynski and Chu, 2001; Trainor et al., 2003). In addition, Marler et al., (1999) found higher levels of AVT immunoreactivity in vocal control centers of satellite cricket frogs (*Acris crepitans*), suggesting that there may be inhibition of AVT secretion from cells in those regions. AVT administration has also been found to elicit calling behavior in one of our study species (*B. cognatus,* Propper and Dixon, 1997) and we have preliminary data suggesting that AVT also reverses satellite expression, consistent with the findings of Propper and Dixon (1997). For example, we have observed rapid (<10 min) elicitation of calling behavior in 4 satellite toads (2 *B. cognatus* and 2 *B. woodhousii*) following administration of 100 μg AVT injections (treated animals were not paired with saline controls). Although Ten Eyck (2005) suggested that the vasotonergic system in calling and satellite Puerto Rican coqui frogs (*Eleutherodactylus coqui*) may differ because of presumed age differences among behavioral phenotypes, there is no evidence to suggest that this is the case with *B. cognatus* or *B. woodhousii*. In these species, satellite males are typically smaller than callers (see also Sullivan, 1982;

Krupa, 1989; Leary et al., 2004) but the behavioral phenotypes do not differ in age (Leary et al., 2005). Rather, skeletochronologic data indicate that the two behavioral phenotypes exhibit different growth trajectories (Leary et al., 2005). We proposed that organizational effects on neural development could occur in early ontogeny to alter activational effects of hormones in adulthood and that presumed differences in age based on body-size differences among behavioral phenotypes in anurans should be reconsidered.

Other hormone manipulation studies in anuran amphibians also indicate that elevated glucocortioids decrease the probability of calling behavior. However, unlike the current results for explosive-breeding toads, the mode of action appears to be through inhibitory effects on circulating androgen levels (Marler and Ryan, 1996; Burmeister et al., 2001). These findings, combined with our present study, raise important questions for studies that alter the social-acoustic environment to examine its influence on reproductive tactic expression in anurans. For example, does the variation in behavioral responses among males exposed to broadcast calls reflect differences in relative attractiveness among those individuals (as predicted by several models, see Introduction) or is the variation in behavioral responses related to differences in the physiological state of the individuals examined? The two perspectives, however, are not mutually exclusive. For instance, it is possible that the physiological state of the individual affects call parameters and relative attractiveness, so that individuals in poor condition produce less attractive calls. For example, Robertson (1986a,b) indicated that the dominant frequency of calls produced by male *Uperoleia rugosa* in good condition may be lower (and more attractive) than calls produced by the same males when in poor condition. Another possibility to consider is that the relative levels of circulating androgens and/or glucocorticoids may be altered during exposure to broadcast calls. For example, Burmeister and Wilczynski (2000) found that exposure to conspecific vocal signals (over a period of several days) results in an increase in circulating androgen and B levels in male green treefrogs (*Hyla cinerea*). Remage-Healey and Bass (*in press*) also found in toadfish (*Opsanus beta*) that exposure to conspecific vocal signals rapidly increases (within minutes) circulating androgen levels, but not glucocorticoid levels. Currently, no study has combined playback studies with endocrine measures to examine the potential interaction between the two factors and their relationship to mating tactic expression in anurans. However, the current investigation and evidence that satellite males are generally in poorer condition than callers (Robertson, 1986a,b; Leary et al., 2004) are consistent with such interactive effects.

The potential endocrine factors regulating vocal behavior in anurans also have important implications regarding satellite-caller dynamic game models. For example, the energetic state of the individual is predicted to be one major component of tactic expression in models proposed by Lucas and Howard (1995), Lucas et al. (1996), and

McCauley et al. (2000). These predictions are consistent with models of endocrine regulation of alternative mating tactic expression in anurans (Emerson, 2001; Leary et al., 2004) given that negative energy balance results in activation of the hypothalamic-pituitary-adrenal axis (resulting in an increase in circulating glucocorticoids, McEwen and Wingfield, 2003). Importantly, however, it is also well established that circulating glucocorticoid levels can be elevated in individuals that are not in a negative-energy state (Sapolsky, 1992a,b). For example, social circumstances could activate the HPA axis independently of the energetic state of the organism (Sapolsky, 1992a,b) thereby altering the probability of satellite tactic expression. In anurans, the potential metabolic costs associated with differences in body size among callers and satellites (Leary et al., 2005), and/or stress associated with being subordinate, may contribute to increased activation of the HPA axis (i.e., see Senar et al., 2000). Hence, investigators finding no differences in measures of relative body condition among behavioral phenotypes should not rule out potential differences in circulating B levels. This may be particularly relevant in anuran species that are highly aggressive and/or territorial.

Given the current evidence, mating tactic expression in anurans is likely to be influenced by interactive relationships between the male’s social environment and physiological state. For instance, Trainor et al. (2003) found that gray treefrogs produced advertisement calls that were longer in duration (i.e., contained more pulses) subsequent to AVT injections, but the response occurred only when individuals were in close proximity to calling conspecific males. Likewise, the outcome of playback studies are likely to be influenced by variability in call parameters as well as the physiological condition of the subjects involved. Currently, playback studies have not controlled for potential physiological differences between the respective phenotypes. However, the potential effects of elevated glucocorticoids on calling behavior highlight that playback experiments, where individuals are often marked (i.e., freeze branded/toe clipped), must be carefully designed and executed because handling and recovery times can dramatically alter circulating glucocorticoid levels (see Moore et al., 1991; Kenagy and Place, 2000).

Satellite behavior is likely to be influenced by additional environmental factors besides the attractiveness of nearby males (i.e., predation risk) and Lucas and Howard (1995) and Lucas et al. (1996) have addressed these issues in stochastic dynamic game models. Such models, when combined with endocrine factors involved in vocal regulation, may provide insight into how different environmental variables act to alter behavioral expression, potentially through a common proximate mechanism. For instance, it is possible that the predicted increase in satellite behavior associated with an increase in predator density (Lucas and Howard, 1995; Lucas et al., 1996) may be mediated by endocrine effects. As an example, blood sucking dipterans (genus *Corethrella*) that use vocal signals to locate potential anuran hosts (McKeever and

French, 1991) could alter circulating B levels (i.e., see Kavaliers et al., 2003) that, in turn, alter mating tactic expression. Similarly, we have repeatedly observed water snakes (*Nerodia erythrogaster*) feeding on *B. cognatus* at one site in central Oklahoma (CJL and AMG, pers. obs.). This site has a high density of water snakes and attempted predation events are frequently unsuccessful when toads are not firmly grasped during the initial strike by the snake. In some cases, toads often struggle for several minutes to escape, but it is not known how such events influence the individual's physiological state. However, such events could lead to rapid elevations in B that alter subsequent behavior.

Current evidence suggests that high glucocorticoid levels inhibit calling behavior in anurans, but that the mode of action may differ in the species examined (i.e., glucocorticoids may act through inhibitory effects on the hypothalamic-pituitary-gonadal axis or through direct effects on neural activity). Unfortunately, comparative data are not currently adequate to ascertain whether such differences among species are related to differences in the temporal frame of breeding activity (explosive versus prolonged breeding species). Regardless, temporal changes in circulating corticosteroid levels associated with reproductive behavior (Emerson, 2001; Romero, 2002; Love et al., 2004; Burmeister and Wilczynski, 2000) and the potential impact of elevated levels B levels on behavioral expression, provide important insights into anuran caller-satellite dynamics that may help explain the behavioral variation observed in vocal playback studies and increase the predictive power of game models.

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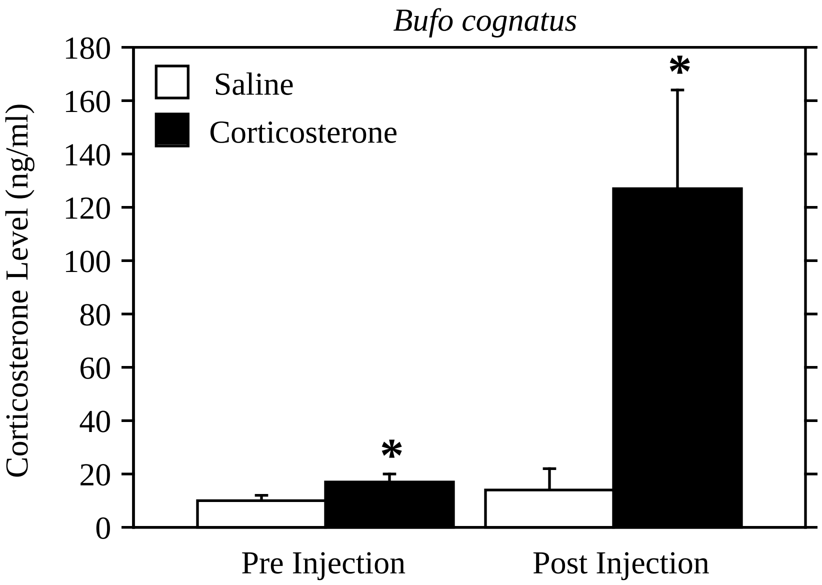
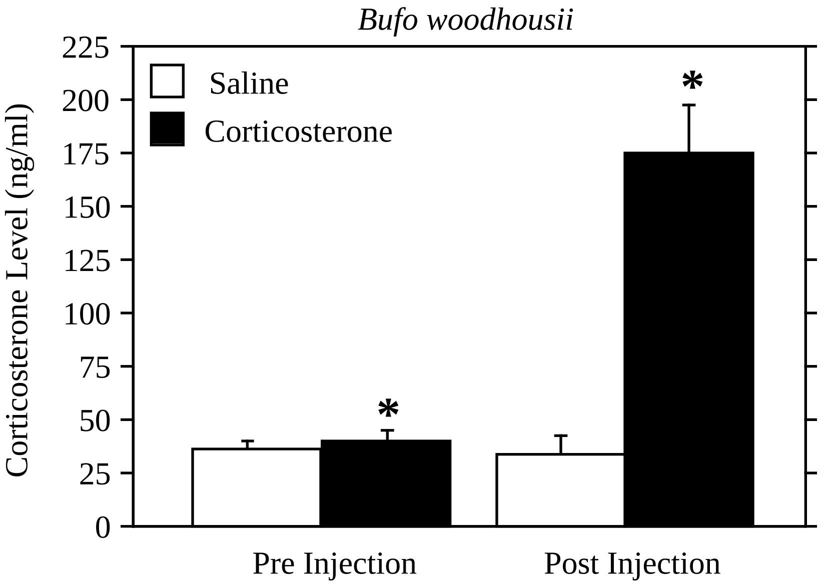
Figure Legends

Figure 1. Mean (±SE) circulating corticosterone levels in male *Bufo cognatus* and *B. woodhousii* before and after saline or corticosterone treatment (significant differences in pre-and post levels are indicated with an asterisk).

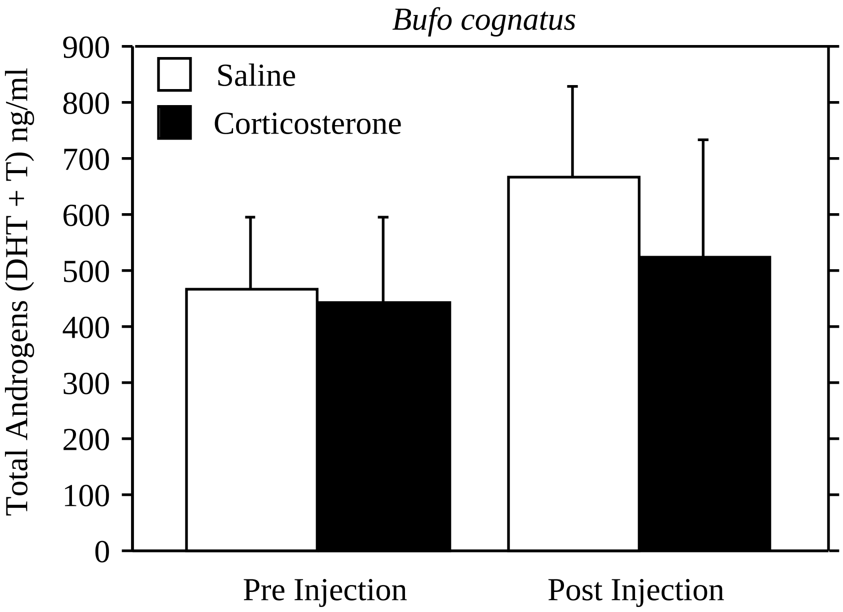
Figure 2. Mean (±SE) circulating androgen levels (dihydrotestosterone + testosterone) in male *Bufo cognatus* and *B. woodhousii* before and after saline or corticosterone treatment.

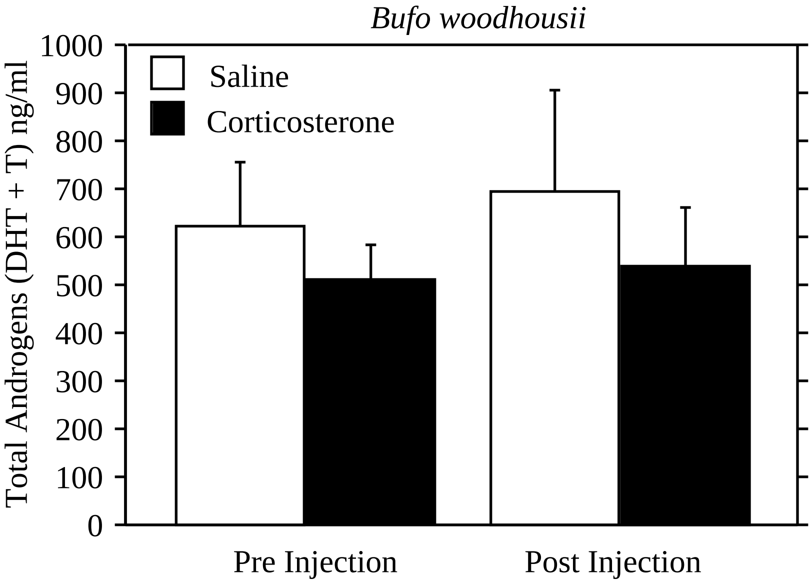


Bufo cognatus
Corticosterone Level (ng/ml)
0
20
40
60
80
100
120
140
160
180
Saline
Corticosterone
*
*
Pre Injection
Post Injection



Bufo woodhousii
Corticosterone Level (ng/ml)
0
25
50
75
100
125
150
175
200
225
Saline
Corticosterone
*
*
Pre Injection
Post Injection


Bufo cognatus
Total Androgens (DHT + T) ng/ml
0
100
200
300
400
500
600
700
800
900
Saline
Corticosterone
Pre Injection
Post Injection


Bufo woodhousii
Total Androgens (DHT + T) ng/ml
0
100
200
300
400
500
600
700
800
900
1000
Saline
Corticosterone
Pre Injection
Post Injection

Chapter III

Hormonal Mediation of a Condition-Dependent Sexually-Selected Trait:

Stress, Attractiveness, and Sexual Parasites

Abstract: Organizational and/or activational effects of gonadal steroids on the expression of sexually dimorphic traits suggest that individual variation in hormone levels can play a major role in sexual selection. Anuran amphibians are ideal organisms in which to examine relationships among male sex steroid levels, signal production, and female preference because vocal production by males is androgen dependent and females often select mates based solely on vocalizations. Here we show that male Great Plains toads (*Bufo cognatus*) in natural choruses producing the longest calls do not have significantly higher levels of circulating androgens than individuals producing shorter calls. Rather, males with the lowest circulating levels of the “stress” hormone corticosterone produce the longest calls that are preferred by females. We show that the effects of experimental elevation of corticosterone levels on call duration are consistent with relationships found under natural conditions; an increase in circulating corticosterone levels resulted in a decrease in call duration, independently of fluctuations in circulating androgen levels. Natural elevations of plasma corticosterone levels appear to be associated with a reduction in males’ relative body condition during vocal production that eventually leads to the cessation of vocalization and the adoption of alternative non-calling “satellite” mating tactics in this species. Our results suggest that selection should favor males with traits that maintain relatively low corticosterone levels or buffer against glucocorticoid-mediated stress responses. A caveat, however, is that in

natural choruses, “host” males with the longest calls and lowest levels of corticosterone are also parasitized by conspecific males practicing the alternative satellite mating tactic. Hence, the most attractive males may be at a reproductive disadvantage relative to other less attractive individuals because of fitness consequences associated with an increased sexual parasite load.

Introduction

Males often vary to a much greater extent than females with respect to conspicuous coloration, morphology, and behavior (Andersson, 1994) and the expression of sexually dimorphic traits is often mediated by organizational and/or activational effects of gonadal steroids (Phoenix *et al*., 1959; Moore, 1991; Ketterson and Nolan, 1999; Ketterson et al., 2001). The implicit role of gonadal secretions in the expression of male-typical traits was historically well recognized (Nelson, 2000) and was the focus of one of the first formal studies in endocrinology (Berthold, 1849). Thus, it is surprising that relatively few studies have established a direct link between circulating sex steroid levels, signal attractiveness, and female preference (reviewed by Enstrom et al., 1997; McGlothlin et al., 2004). This apparent discrepancy may lie in the complexity of endocrine mediation of male-typical traits that could involve effects of hormones other than sex steroids (e.g., Moore and Evans, 1999), variation in ligand specific receptor levels (e.g., Young, 1999), and/or variation in binding globulin levels (reviewed by Breuner and Orchinik, 2002).

Vocal production in anuran amphibians is a well-known sexually dimorphic androgen-dependent behavior (reviewed by Kelley, 2004). In Great Plains toads (*Bufo cognatus*), males alternate between one of two reproductive tactics: 1) individuals may vocalize to attract mates or 2) they may adopt silent “satellite” tactics wherein males position themselves in close proximity to calling conspecific males and attempt to intercept incoming females attracted to the vocalizations of calling “hosts” (Leary *et al*., 2004; see Halliday and Tejedo, 1995 for review of alternative mating tactics in anurans). Behavioral transitions from calling to non-calling satellite tactics in this species are mediated by an increase in circulating corticosterone levels associated with a reduction in body mass during vocal production (Leary *et al*., 2004; Leary *et al*., in review b) and is not dependent on an individual’s age (Leary *et al*., 2005). Natural circulating levels of glucocorticoids are not inversely correlated with circulating androgen levels in this species, and corticosterone injections initiate rapid (< 1hr) behavioral transitions from calling to satellite behavior independently of changes in androgen levels, suggesting that corticosterone may act via non-genomic pathways to inhibit the secretion of the neuropeptide arginine vasotocin in brain vocal control centers (Leary *et al*., 2004; Leary *et al*., in review a,b).

In the current series of studies, we establish a direct causal relationship between circulating corticosterone levels, signal attractiveness, and mate selection in Great Plains toads. We show that: 1) satellite males in natural choruses associate with vocalizing conspecific host males producing the longest calls, 2) males with associated satellites in natural choruses have significantly lower cortisosterone levels than males without associated satellite males, 3) corticosterone injections decrease call duration in males, and 4) in dual speaker playback experiments, females prefer calls of longer duration produced by males with low corticosterone levels.

Our results show that corticosterone, a hormone whose secretion is elicited under various stressful conditions, can have a profound effect on male traits selected by females and, at the same time, used by satellite males to determine which calling male they parasitize. Hence, while non-calling males potentially optimize their reproductive success by associating with calling males with the longest calls and lowest corticosterone levels, parallel preferences between satellite males and females potentially poses increased fitness risks for attractive calling males.

Methods and Results

Vocal attributes of males with and without associated satellite males in natural choruses

A. Methods

A series of advertisement vocalizations were recorded from calling male *B. cognatus* with associated satellite males and nearby calling males without satellite males in natural choruses during peaks in reproductive activity (following heavy rains in April-June 2001-2004) in central Oklahoma. Choruses consisted largely of *B. cognatus* only (i.e., chorus frogs, *Pseudacris clarkii,* were the only other anuran species present at our study sites during the investigation). We observed undisturbed behavior of individuals for 15 to 30 min to ensure accurate categorization of behavioral phenotypes and satellite-caller associations prior to collecting data. Observations were made under ambient light conditions or with low powered LED headlamps and did not have any overt effects on behavior. Vocal recordings were made using a Marantz PMD 222 recorder equipped with a Sennheiser ME66 condenser microphone and Sennheiser K6-C power module. Call amplitude was measured with a RadioShack digital sound pressure level (SPL) meter (Model 33-2055) with a 31 cm heavy gauge wire mounted to the face to ensure that the distance between the subject and SPL meter was consistent between measures. Calling individuals were approached and the wire mount on the SPL meter was oriented horizontal to the water substrate and placed directly in front of (almost touching) the calling male's expanded gular sac. Calls were never less than 11.3 sec in duration, allowing accurate SPL levels to be obtained over a series of calls. Call amplitude was obtained from a subset of the same individuals for which recordings were made.

Calls were analyzed using Canary 1.1.1 Bioacoustics software (settings: frame length 256 points, time 5.752 ms, 50% overlap, fast Fourier transform size 256 points, Hamming filter and amplitude logarithmic). We examined call parameters known to be important in mate selection in bufonids and other anurans (reviewed by Gerhardt and

Huber, 2002) including call duration, pulse rate (defined throughout as the number of pulses in a 3 sec section from the middle portion of the call), dominant frequency, and intercall duration measured from spectrograms, spectra, and waveforms for 5 to 10 consecutive calls from each individual.

Temperature effects on call parameters are well known in anurans (Gerhardt and Huber, 2002). All *B. cognatus* males at our study sites called while partly submerged in water and water temperature was nearly identical to cloacal body temperatures (CJL, *pers. obs*.). *Bufo cognatus* bred in shallow, small ephemeral pools, with consistent water temperatures within sites and within nights of data collection. Water temperature across sites and years never varied more than 5ºC (range 20-25ºC). Data from callers with associated satellites was always collected in conjunction with data collected from nearby males (within a 3 m radius) without associated satellites males. Males without associated satellites outnumbered males with associated satellites in the given area and thus, ANCOVA (with temperature as the covariate) were done on unequal numbers of callers with and without associated satellites.

B. Results

There was no significant difference in temperature for males with and without associated satellite males ($F_{1,57} = 1.3$, $p = 0.25$). With the exception of pulse rate, there was no significant interaction (heterogeneity of slopes) for the effects of temperature on call parameters for callers with and without associated satellite males ($p > 0.15$). Hence, the interaction term was removed from the analyses for call duration, dominant frequency, intercall duration, and amplitude, and the models were rerun. Heterogeneity of slopes for the effects of temperature on pulse rate for callers with and without associated satellite males precluded interpretation of the main effects from ANCOVA (Hendrix et al., 1982). We followed the statistical procedures outlined by Hendrix et al. (1982) and Tomkins and Simmons (2002) to examine potential differences in pulse rates for callers with and without associated satellites. Briefly, this statistical procedure entailed adjusting the pulse rate to the mean covariate value (23.2ºC) and1 SD below (21.6ºC) and 1 SD above (24.8ºC) the mean temperature value using pooled regressions for the effects of temperature on pulse rate for callers with and without associated satellite males. ANOVA were then run on adjusted values at each temperature.

Mean dominant frequency, intercall duration, and amplitude did not differ between callers with and without associated satellites (Table 1). However, calling males with associated satellites produced advertisement vocalizations that were significantly longer in duration and had lower pulse rates than those produced by nearby callers without associated satellites (Fig 1; Table 1), suggesting that satellite males preferentially associate with calling males producing the longest calls and/or with low pulse rates.

Hormone levels of satellite males and males with and without associated satellite males

A. Methods

Blood samples were obtained by cardiac puncture from callers and their associated satellites, and nearby calling males without associated satellites, immediately after vocal recordings were obtained. Sample collection never took more than 30 sec and blood samples obtained in the field were kept on ice until they were returned to the lab (< 4 hours) and centrifuged for 12 min at 3000 rpm. Plasma was collected and stored at 20°C until it was assayed for steroid hormones. All sampled individuals were numerically marked on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL) for future identification. All procedures were approved by the University of Oklahoma Animal Care and Use Committee (#A3240-01).

Hormone extraction, chromatography, and radioimmunoassay followed the methods described in Leary *et al*. (2004) and were validated in this previous investigation. Briefly, plasma samples were extracted with diethyl ether, dried under nitrogen gas, and resuspended in 10% ethyl acetate in iso-octane. Column chromatography was then used to separate dihydrotestosterone (DHT), testosterone (T), and corticosterone (B). Testosterone antibody was obtained from Research Diagnostics (Flanders, NJ) and used for both T and DHT assays. Corticosterone antibody was purchased from ICN Laboratories (Costa Mesa, CA).

B. Results

ANOVA revealed a significant overall difference in circulating B levels among groups ($F_{2,89}$ = 11.5, *p* = 0.0001; Fig 2A). Calling males without associated satellite males had significantly higher B levels than calling males with satellite males (Scheffe's post-hoc test, *p* = 0.01) and satellite males (*p* = 0.0001). There was a trend for calling males with associated satellite males to have higher B levels than satellite males (*p* = 0.06). Sample sizes differ from those reported for call attributes because we were not able to obtain blood samples from all individuals that were recorded and analyses included additional individuals for which calls were not obtained.

Circulating levels of androgens (DHT + T) did not differ for calling males with and without associated satellites or satellite males (overall ANOVA $F_{2,89}$ = 2.0, *p* = 0.14; Fig 2B).

Effects of exogenous corticosterone on call parameters

A. Methods

Corticosterone was administered to calling males to establish a causal relationship between B and call parameters. Hormone manipulation procedures are described in a previous study in which we assessed the effects of exogenous B on mating tactic expression (Leary *et al.*, in review b). Briefly, calling male toads were administered saline or B in saline vehicle and subsequently monitored for mating and calling behavior. Estimated dosages were based on circulating B levels and body mass measures for this species ascertained in our previous investigation (Leary *et al*., 2004). Crystalline B (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol (60 μg B/0.1 ml EtOH), evaporated, and resuspended in a saline solution vehicle (115m*M* NaCl, 2.5 m*M* $KHCO_3$, 1.0 m*M* $CaCl_2$, see Propper and Dixon, 1997). Toads were administered a dose of approximately 0.81 μg B/g body mass based on the mean body mass (73.7 g) of *B. cognatus* males used in this study. Controls were administered an equal volume (100 μl) of saline vehicle only.

Vocalizations of calling male toads were recorded in natural choruses for approximately 10-20 min prior to treatment. Calling individuals were then injected in the intraperitoneal cavity in a paired fashion; one male was injected with 100 μl B and one male was injected with 100 μl saline. In a single case, two B-treated individuals were paired with a single saline-injected male. Thus, we examined the effects of treatment from a total of 9 individuals (5 B-injected individuals and 4 saline-injected individuals). All individuals continued to call or resumed calling within 1 min after treatment.

Calls were continuously recorded subsequent to treatment until satellite behavior was adopted (for at least 5 min) or after 1 hour (Leary *et al.*, in review b). Males were recaptured at this time and another blood sample was obtained (see Leary *et al*., in review b). Corticosterone administration consistently elevated B above pre-administration levels (pre-B injection mean = 16.59 ng/ml, SE = 3.89, range = 5.18 - 29.15 ng/ml; post-B injection = 127.43 ng/ml, SE = 36.59, range = 9.28 – 199.48 ng/ml). Corticosterone injections had no effect on circulating androgen levels ($p = 0.3$) and saline-injected controls did not show a significant change in B ($p = 0.4$) or androgen levels ($p = 0.2$, see Leary *et al*., in review b). We measured call charcteristics of 6-10 calls from each male at three times: prior to treatment, within 10 min post-treatment, and approximately 30 min post-treatment.

B. Results

Repeated measures ANOVA indicated no overall between-groups difference in call duration ($F_{1,7} = 1.8$, $p = 0.21$) and no significant main effect of time on call duration ($F_{1,2} = 1.0$, $p = 0.36$). However, there was a decrease in call duration over time for B-treated but not saline-injected individuals, and a significant interaction between B-injected and saline-injected groups (group x time interaction: $F_{1,2} = 6.6$, $p = 0.009$; Fig 3A).

There was no overall between-groups difference in pulse rate ($F_{1,7} = 0.1$, $p = 0.71$). However, there was a significant decrease in pulse rate over time ($F_{1,2} = 8.4$, $p =$

0.004), but no significant difference in the response of B-injected and saline-injected groups ($F_{1,2}$ = 2.2, *p* = 0.14; Fig 3B).

Corticosterone administration affected intercall duration in a similar manner as call duration. There was no overall difference between groups ($F_{1,7}$ = 0.1, *p* = 0.74) and no overall effect of time on intercall duration ($F_{1,2}$ = 0.09, *p* = 0.91), but B and saline-treated individuals exhibited different responses over time ($F_{1,2}$ = 5.3, *p* = 0.01; Fig 3C).

There was no overall group difference in dominant frequency ($F_{1,7}$ = 4.8, *p* = 0.06), no temporal main effects across groups ($F_{1,2}$ = 1.2, *p* = 0.33), and no significant difference in the response over time between B-injected and saline-injected groups ($F_{1,2}$ = 0.5, *p* = 0.61).

Female preference for calls produced by males with low and high corticosterone levels

A. Methods

The association between satellite males and hosts with the lowest B levels appeared to be related to the effects of B on call duration. Although pulse rate was also significantly different for callers with associated satellite males and callers without associated satellites (Fig 1), there was no detectable effect of B on pulse rate (Fig. 4B). Furthermore, pulse rate is typically under stabilizing selection in bufonids and other anurans (i.e., values near the mean are most attractive, Gerhardt and Huber, 2002). Call duration, however, is typically under strong directional selection in anurans, with females preferring calls of longer duration (Gerhardt and Huber, 2002). Because call duration was the only call parameter to consistently appear as an important factor in the above investigations, we focused on this call parameter to determine if satellite males potentially maximize their chances of intercepting a mate by associating with conspecific calling host males producing the most attractive (i.e., longest) calls.

We, therefore, assessed whether females preferentially selected calls produced by males with associated satellite males versus calls produced by males without associated satellite males. We performed dual speaker playback experiments wherein calls were broadcast to gravid females. Parameters of broadcast calls from the two speakers were identical for all parameters except call duration. Call parameters used in playback studies were based on the mean values for 24 males recorded from several choruses in central Oklahoma in 2001 (recorded at an average temperature of 24°C; pulse rate = 55 pulses/3 sec, dominant frequency = 2.2 kHz, intercall duration = 9.75 sec, amplitude from 31 cm = 103 dB). Differences in call duration used in playback experiments were based on the mean duration from preliminary data collected in 2001 for calling males with (n = 12) and without satellite males (n = 9), respectively. These values closely approximated differences with a larger sample size shown in Figure 1A and Table 1 [one speaker broadcast calls 22.5 sec in duration and the other broadcast calls 17.5 sec in duration].

Calls used in playback experiments were taken from recordings of a single individual male that produced calls with characteristics representative of the mean values from the 2001 data set (described above). A single call from that individual was digitized using Canary 1.2.1 software. Call duration was altered by removing a series of pulses from the middle portion of the original call or adding pulses to the middle portion of the call while maintaining constant pulse rate/periodicity. Call duration manipulation inevitably alters other call characteristics (e.g., total number of pulses are greater for long calls versus short calls, and call rate increases for calls of short duration). However, playback stimuli were representative of differences in call duration for males with and without associated hosts (Table 1) or with low and high B levels (Fig 1A).

Digitized calls were imported into MacIntosh iTunes from Canary AIFF files and a continuous loop playback of the digital file was transferred to cassette tapes used in playback trials. Gravid females collected at natural choruses were brought to the lab within 4 hours after capture and run in preference trials. A female was placed under a cloth box in the center of the chamber for 5-10 min while exposed to broadcast calls. Calls were broadcast from Sennheiser accessory speakers connected to the Marantz recording equipment that were placed 3.05 m apart in a 3.2 m X 2.1 m semi-anechoic chamber. The box was then lifted with a pully mechanism when the onset of broadcast calls were synchronized (calls were subsequently out of synchrony because of differences in call duration). Chamber temperature was held at the mean temperature of natural chorus activity (24°C) and broadcast call amplitude was checked prior to trials with an SPL meter and held at a constant 103 dB. Behavioral responses were scored under red incandescent light by an observer seated behind a blind. Positive phonotactic scores included female orientation, approach, and direct contact with the speaker. Cassette tapes were switched between speaker playback systems between successive trials to eliminate extraneous factors that potentially influence movement patterns.

A total of 14 females were run in playback trials. Two females were unresponsive to calls and attempted to escape. Of the 12 females that responded, 6 were run in 2 trials and 6 were run in a single trial. All 12 females responded within 5 min and 100% preferred the speaker broadcasting the longer calls in all trials; all females run in two trials preferred the calls of longer duration in both trials. Hence, females had a strong preference for the longer calls (n = 12 females (12 trials), $X^2 = 12$, $p < 0.001$) characteristic of a calling male with low B levels.

Discussion

Our results demonstrate that differences in circulating levels of the stress steroid corticosterone can have a dramatic effect on signal attractiveness and mate acquisition in

Great Plains toads. These results differ substantially from previous studies on various bird species that have focused on the effects of elevated testosterone on male mating success (reviewed in McGlothlin et al., 2004) because differences in male attractiveness in Great Plains toads were not related to differences in circulating androgen levels. Hence, there are fitness consequences associated with elevated corticosterone levels in male toads that act independently of fluctuations in gonadal steroids.

Our work on male Great Plains toads and Woodhouse's toads (*Bufo woodhousii*) has shown that chorusing males experience a reduction in body mass that appears to drive an increase in circulating corticosterone levels (Leary et al., 2004). Elevations in corticosterone mediate transitions from calling to non-calling satellite mating tactics independently of fluctuations in circulating levels of androgens in these species (Leary *et al*., 2004; Leary et al., in review b). The present study shows that variation in circulating corticosterone levels among chorusing male Great Plains toads directly affects male signal attractiveness and satellite-caller associations. Calling males with associated satellite males do not, however, have significantly different circulating levels of androgens than calling males without associated satellite males. Rather, males with the highest levels of circulating B in natural choruses produced calls that were significantly shorter in duration than males with low levels of circulating B. Corticosterone administration resulted in a significant reduction in call duration compared to control injections. However, experimental elevation of B does not have a reciprocal effect on androgen production (Leary *et al*., in review b). Because of the rapid effects of B on mating tactic expression (Leary *et al*., 2004; Leary *et al*., in review b) and call characteristics, B may act through non-genomic pathways that alter the secretion of arginine vasotocin in vocal control centers of the brain (e.g., see Orchinik et al., 1991; Moore and Evans, 1999).

Our combined investigations on *B. cognatus* indicate that natural elevation in B during chorus activity result in a progressive decrease in call duration that eventually leads to the cessation of calling activity and the expression of alternative non-calling reproductive tactics. Females prefer the calls characteristic of males with the lowest B levels, suggesting that selection may favor any heritable component associated with maintenance of relatively low corticosterone levels or suppression of the stress response (e.g., binding protein levels, receptor levels, metabolic rates, growth rates, body size, foraging efficiency). However, sexually-parasitic males practicing alternative reproductive tactics also associate with calling males with the lowest B levels that produce the longest calls. Sexual parasite load is known to vary considerably in *B. cognatus*, with calling host males having 1 to 5 associated satellite males (Sullivan, 1982; Krupa, 1989; CJL and AMG, *pers obs*.). Hence, although calling males in the best physical condition with the lowest levels of circulating corticosterone produce the most attractive calls, our results suggest that males with the longest calls may also experience a reduction in reproductive success by attracting sexually parasitic conspecific satellite males — a diminishing returns effect (Waltz, 1982). Hence, directional selection on preferred male traits via female choice may be countered by host selection via males practicing alternative mating tactics and appears to be mediated by a hormone reflecting the calling males' physiological condition.

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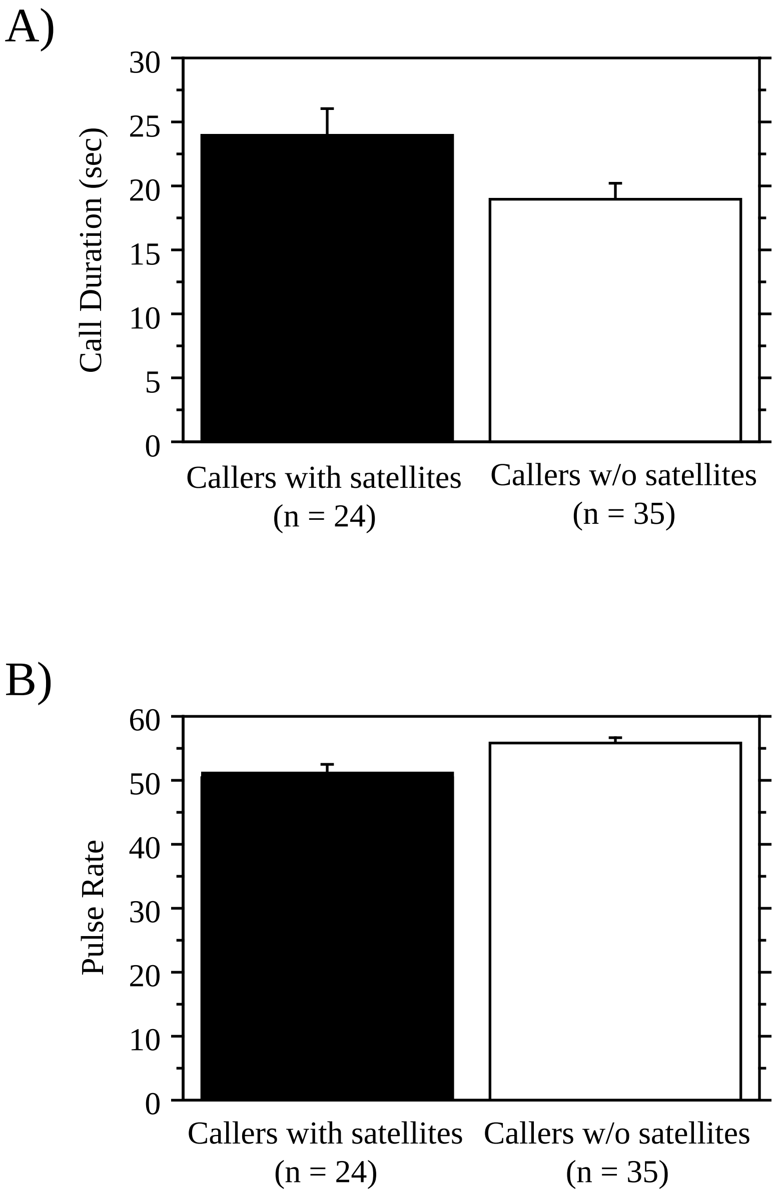
Figure Legends

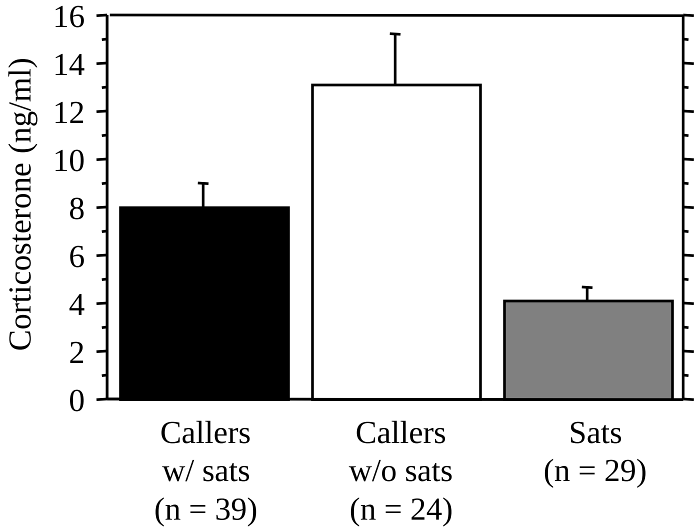
Figure 1. Mean (± SE) call duration (A) and pulse rate (B) values for calling male *B. cognatus* with associated satellite males and without associated satellite males in natural choruses. Pulse rate values were adjusted to the mean temperature value (23.2ºC). Both parameters are significantly different between the two groups (see text).

Figure 2. Mean (± SE) circulating corticosterone (A) and total androgen (dihydrotestosterone + testosterone) levels for calling males with associated satellite males, without associated satellite males, and satellite males from natural choruses (see text for statistics).

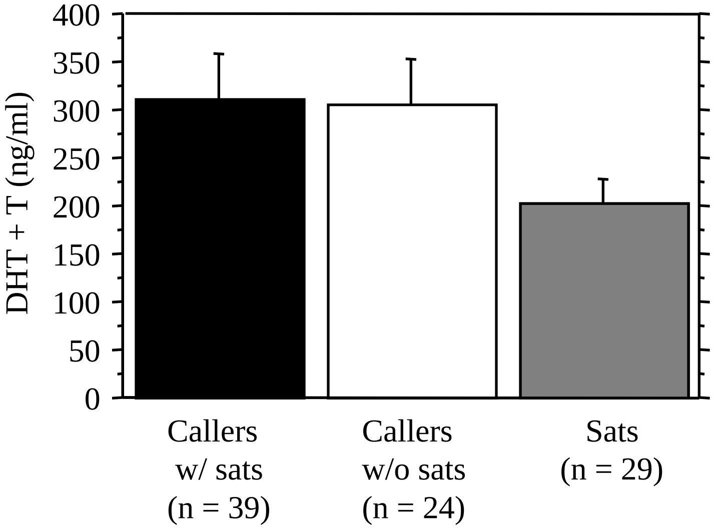
Figure 3. Interaction plots (mean± SE) for the effects of corticosterone and saline injections on call duration (A), pulse rate (B), and intercall duration (C) over three periods of sampling (Pre = prior to treatment, Post 1 = <10 min post injection, Post 2 = ~30 min post injection, see text for statistics).


A)
Call Duration (sec)
30
25
20
15
10
5
0
Callers with satellites
(n = 24)
Callers w/o satellites
(n = 35)
B)
Pulse Rate
60
50
40
30
20
10
0
Callers with satellites
(n = 24)
Callers w/o satellites
(n = 35)

A)


Corticosterone (ng/ml)
16
14
12
10
8
6
4
2
0
Callers w/ sats (n = 39)
Callers w/o sats (n = 24)
Sats (n = 29)

B)


DHT + T (ng/ml)
400
350
300
250
200
150
100
50
0
Callers w/ sats (n = 39)
Callers w/o sats (n = 24)
Sats (n = 29)

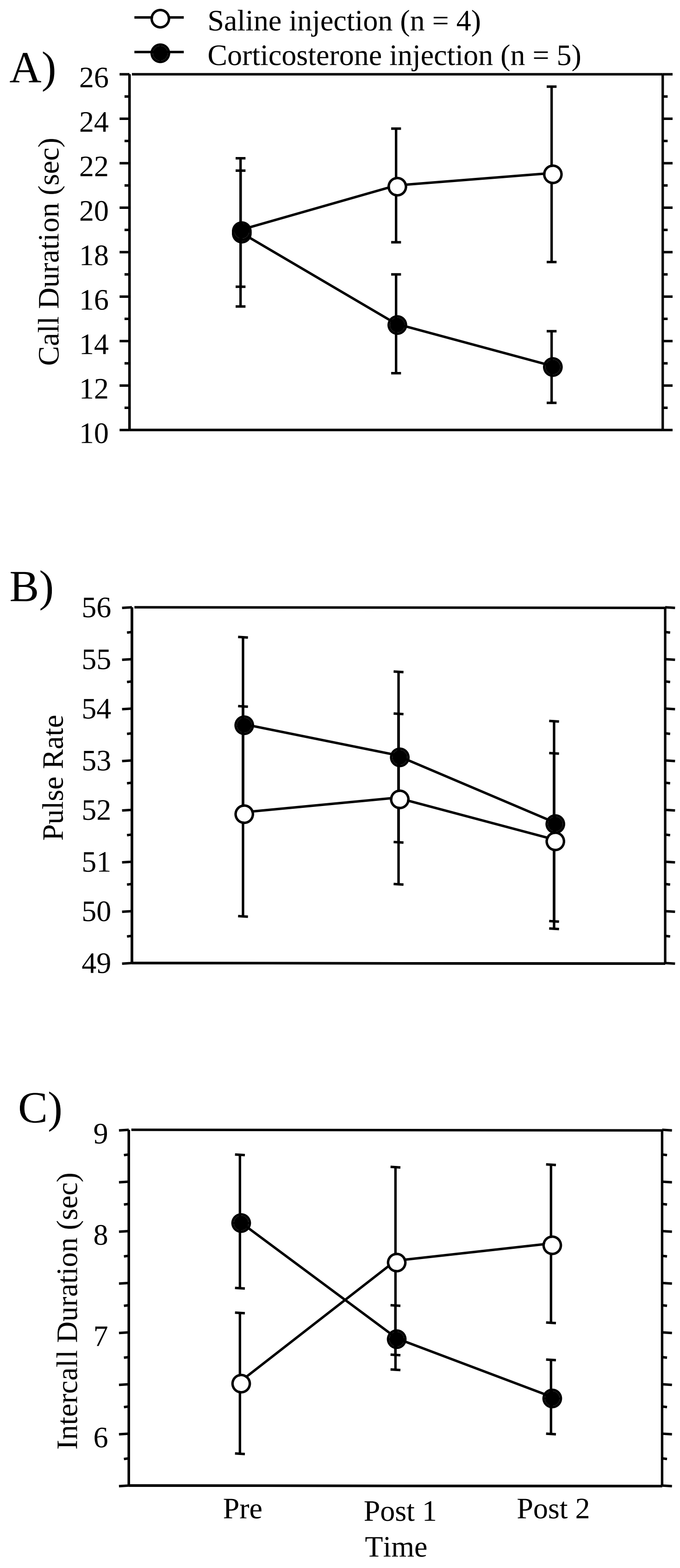

Saline injection (n = 4)
Corticosterone injection (n = 5)
A)
Call Duration (sec)
26
24
22
20
18
16
14
12
10
B)
Pulse Rate
56
55
54
53
52
51
50
49
C)
Intercall Duration (sec)
9
8
7
6
Pre
Post 1
Post 2
Time

Table 1. Mean (±SE) values for call parameters of calling male *Bufo cognatus* with associated satellites and without associated satellites.

Call Parameter	Caller with Satellite	Caller without Satellite	ANOVA Results
Call Duration (sec)	24.2 (±1.6)	18.8 (±1.3)	$F_{1,56} = 4.94$, $p = 0.03$
Pulse Rate (number of pulses in 3 sec)			
a. Adjusted to mean temperature (23.2ºC)	51.1 (±0.9)	55.8 (±0.8)	$F_{1,57} = 14.83$, $p = 0.0003$
b. Adjusted 1 SD below the mean (21.6ºC)	48.8 (±0.9)	51.1 (±0.8)	$F_{1,57} = 3.73$, $p = 0.06$
c. Adjusted 1 SD above the mean (24.8ºC)	53.5 (±0.9)	60.5 (±0.8)	$F_{1,57} = 33.29$, $p = 0.0001$
Dominant Frequency (kHz)	2.2 (±0.04)	2.2 (±0.03)	$F_{1,56} = 0.20$, $p = 0.66$
Intercall Duration (sec)	10.3 (±1.4)	9.2 (±0.9)	$F_{1,56} = 0.66$, $p = 0.42$
Amplitude (dB)	103.7 (±2.8)	103.5 (±3.2)	$F_{1,45} = 0.05$, $p = 0.82$