

# Social Context Influences Androgenic Effects on Calling in the Green Treefrog (*Hyla cinerea*)

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Courtship behavior in frogs is an ideal model for investigating the relationships among social experience, gonadal steroids, and behavior. Reception of mating calls causes an increase in androgen levels in listening males, and calling, in turn, depends on the presence of androgens. However, previous studies found that androgen replacement does not always restore calling to intact levels, and the relationship between androgens and calling may be context dependent. We examined the influence of androgens on calling behavior in the presence and the absence of social signals in male green treefrogs (*Hyla cinerea*). We categorized calling during an acoustic stimulus (mating chorus or tones) as evoked and calling in the absence of a stimulus as spontaneous. Intact males received a cholesterol implant, castrated males were castrated and received a cholesterol implant, and T-implanted males were castrated and received a testosterone implant. The androgen levels (mean  $\pm$  SE ng/ml of plasma) achieved by the implants were as follows: castrated males,  $1.2 \pm 0.2$ ; intact males  $21.9 \pm 7.0$ ; T-implanted males,  $254.6 \pm 39.5$ . As in other frogs, calling depends on the presence of androgens, as castration abolished and T replacement maintained calling. However, among intact and T-implanted males, the influence of androgens on calling differed between spontaneous and evoked calling. There was a positive effect of androgen treatment on spontaneous call rate and a positive correlation between spontaneous call rate and androgen levels. The influence of androgen levels on evoked call rate was more complex and interacted with acoustic treatment. Surprisingly, T implants suppressed the chorus-specific increase in calling that is evident in intact males. In addition, in response to the chorus, T-implanted males called less than did intact males, in spite of higher androgen levels. Furthermore, variation in androgens did not explain variation in evoked call rate.

These data indicate that androgens influence the motivation to call, but that, when socially stimulated, androgens are necessary but insufficient for calling.

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The field of behavioral endocrinology was established with the demonstration that sex-typical behaviors are dependent on gonadal steroids (Berthold, 1849), and this principle has subsequently been applied to a wide array of vertebrates. However, there is also tremendous diversity in hormone–behavior relationships (Crews and Moore, 1986), and the influence of steroids on behavior often depends on the physiological and social context in which they occur. This is true in the simple sense that androgens, for instance, may promote courtship in the presence of females and aggression in the presence of males. But context may also have more subtle effects. For example, exposure to male song may increase the effectiveness of estradiol in producing nest building behavior in female birds (Hinde and Steel, 1978). Perhaps more dramatically, social experience may cause copulation to persist after castration in rodents (Meisel and Sachs, 1994) and lizards (Sakata, Gupta, and Crews, 2001). Social regulation of the neural structures underlying hormone-dependent behaviors (Hartman and Crews, 1996; Tramontin, Wingfield, and Brenowitz, 1999) is a potential mechanism by which social signals may influence hormone–behavior relationships. Furthermore, the relationship between sex steroids and behavior becomes more complex when one considers that behavior can have pronounced effects on reproductive physiology; for example, reception of social cues regulates timing of puberty (Rissman, 1992), ovulation (Cheng, 1986; Stern and McClintock, 1998), and

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secretion of gonadal steroids (Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001).

Courtship behavior in frogs is an ideal model for investigating the relationships among social experience, steroids, and behavior for several reasons. First, courtship behavior in frogs is highly social. Males gather in lek-type aggregations and call to attract females. Mating success is highly skewed and the most important determinant of mating success is the number of nights a male spends calling (Dyson, Henzi, Halliday, and Barrett, 1998; Gerhardt, 1987; Ryan, 1985). Second, the reception of social signals evokes both behavioral and endocrine responses in listening males (Burmeister and Wilczynski, 2000), demonstrating an important connection between the communication and endocrine systems. Finally, calling is also androgen dependent. The vocal production pathway concentrates sex steroids (Kelley, 1981; Kelley, Morrell, and Pfaff, 1975; Morrell, Kelley, and Pfaff, 1975) and calling depends on the presence of androgens (Wada and Gorbman, 1977; Wada, Wingfield, and Gorbman, 1976; Wetzel and Kelley, 1983). In addition, seasonal changes in androgen levels track seasonal changes in calling (Gobbetti, Zerani, Bolelli, and Botte, 1991; Harvey, Propper, Woodley, and Moore, 1997; Itoh, Inoue, and Ishii, 1990; Itoh and Ishii, 1990; Polzonetti-Magni, Mosconi, Carnevali, Yamamoto, Hanaoka, and Kikuyama, 1998; Varriale, Pierantoni, Di Matteo, Minucci, Fasano, D'Antonio, and Chieffi, 1986). However, the relationship between androgen levels and calling during the breeding season remains somewhat unresolved: androgens are lower in calling than in noncalling toads (Mendonça, Licht, Ryan, and Barnes, 1985; Orchinik, Licht, and Crews, 1988), but higher in calling coqui (Townsend and Moger, 1987) and túngara frogs (Marler and Ryan, 1996). Further, there is support for a positive relationship between androgen level and call motivation, as measured by evoked call rate (Solís and Penna, 1997) and latency to resume calling after capture (Burmeister, Somes, and Wilczynski, 2001).

The fact that call reception causes changes in androgen levels (Burmeister and Wilczynski, 2000) suggests that correlations between androgens and calling could be the product of being in a chorus and does not necessarily support a causal effect of androgens on calling. In fact, studies which examine calling in response to hormone manipulation demonstrate that androgen replacement following castration does not always restore/maintain calling at intact levels (Palka and Gorbman, 1973; Schmidt, 1966; Wada and Gorbman, 1977; Wetzel and Kelley, 1983). Treatments with

pituitary tissue (Palka and Gorbman, 1973; Wada *et al.*, 1976) or gonadotropins (Wetzel and Kelley, 1983) are sometimes more effective in restoring calling. Furthermore, not only does hearing calls increase androgens, but the relationship between androgens and calling may be context dependent. In a previous study, calling rate was not correlated with plasma androgen levels in socially stimulated males, whereas androgen levels were higher in spontaneously calling males who were not socially stimulated than in noncallers (Burmeister and Wilczynski, 2000). This suggests that androgens may influence the motivation to call, but that social stimulation may eliminate or mask the relationship between calling and androgens. In order to address these issues, we examined the influence of androgens on calling behavior in the presence and the absence of social signals.

## MATERIALS AND METHODS

In order to assess the role of androgens on calling, we manipulated gonadal steroids and exposed male treefrogs to either a mating chorus or tones, resulting in six treatment groups (Hormone (3 levels)  $\times$  Stimulus (2 levels)). We presented males with one of two acoustic stimuli for 5 h each night, and we measured call rate throughout each of 7 days of acoustic treatment. As a result, for each individual, we measured call rate in the presence and the absence of an acoustic stimulus.

### *Hormone Manipulation*

Male *Hyla cinerea* were purchased from Charles Sullivan Company (Nashville, TN) and housed in groups prior to acoustic treatment. Individuals were randomly assigned to one of three hormone treatments: intact males ( $n = 26$ ) received a cholesterol implant, castrated males ( $n = 24$ ) were castrated and received a cholesterol implant, and T-implanted males ( $n = 21$ ) were castrated and received a testosterone implant. We created steroid implants by filling silastic capsules (0.058 in. i.d., 0.077 in. o.d., 6.5 mm total length) with approximately 6.9 mg/implant of testosterone (T) or 4.2 mg/implant of cholesterol. The actual levels of androgens (mean  $\pm$  SE ng/ml of plasma) achieved by the implants were as follows: castrated males,  $1.2 \pm 0.2$ ; intact males,  $21.9 \pm 7.0$ ; T-implanted males,  $254.6 \pm 39.5$ . Although we implanted only T, pilot studies indicate that treefrogs metabolize implanted T into dihydrotestosterone in proportion to T, whereas

estradiol levels remain undetectable (Burmeister and Wilczynski, unpublished data). Prior to surgery, males were anesthetized by placing them in a 2.5% aqueous solution of tricaine methyl sulfonate (Sigma-Aldrich, Milwaukee, WI) for approximately 3 min. All animals underwent surgery and received a small ventral incision; wounds were sealed with a combination of sutures and Vetbond (World Precision Instruments, Sarasota, FL). Animals were allowed to recover for 3–6 days prior to acoustic treatment.

### *Acoustic Treatment*

General procedures for acoustic treatment followed those described in Burmeister and Wilczynski (2000). We housed males individually in chambers (internal dimensions 14 × 14 × 20 cm), which were equipped with a speaker, microphone, water dish, artificial foliage, and light source (14:10 light:dark). Individuals were exposed for 7 nights to either a mating chorus or an array of tones (stimuli described in more detail in Burmeister and Wilczynski, 2000). The chorus was a 12-min recording of a naturally breeding population. We created the tone stimulus by replacing every frog call in the chorus with a tone of the same duration and approximate amplitude as the call it replaced. Tones were within the hearing range of the species, but excluded frequencies that are important in conspecific communication. Stimuli played for 5 h from 2100 to 0200 h (stimulus onset 1 h after lights out). Calling behavior of individuals was monitored by a computer that received input from the chamber microphones, using software written for the study. Given a limitation on the number of acoustic chambers, a subset of subjects was treated (3 to 4 from each treatment group) sequentially until completion (four iterations). On the seventh day, we collected trunk blood with heparinized capillary tubes following rapid decapitation. Blood was centrifuged, and plasma stored at –20°C until radioimmunoassay.

### *Calling Behavior*

A computer monitored calling behavior continuously throughout the experiment, counting the number of calls produced in each 30-min period. In the laboratory, green treefrogs call spontaneously at low rates during morning hours, but generally do not call at night unless presented with a mating chorus (Burmeister and Wilczynski, 2000). Therefore, we defined two classes of calling behavior. The spontaneous calling rate was defined as the mean number of calls per

hour produced in the absence of an acoustic stimulus (0200–2100 h). The stimulus-evoked calling rate was defined as the mean number of calls per hour produced during the acoustic stimulus period (either chorus or tones, 2100–0200 h). The mating chorus successfully evokes calling under these conditions, whereas tones do not elicit calling more than no sound (Burmeister and Wilczynski, 2000). Regardless of the stimulus, we refer to calling during this period as “evoked.” We averaged evoked and spontaneous call rates over the 7 days of the experiment, as the number of days of stimulus exposure did not influence call rate in this or a previous study (Burmeister and Wilczynski, 2000). We also examined the mean number of calls produced during each hour of the day in order to examine the diurnal pattern of calling. Finally, an individual male was defined as a caller if he produced one or more calls at any point during the experiment. As a result, a caller may have a zero call rate within one of the two defined periods.

### *Radioimmunoassay*

A commercially available radioimmunoassay (RIA) kit for androgens (TRK-600, Amersham Pharmacia Biotech, Piscataway, NJ) was used to measure androgens as follows (see below for validation of the procedure). In order to calculate recoveries for individual samples (mean 86%), we added approximately 2500 cpm of <sup>3</sup>H-labeled testosterone to each sample prior to ether extraction. Steroids were extracted from plasma with diethyl ether (2 × 3 ml), and ether fractions were collected after freezing water fractions in an acetone-dry ice bath. Ether fractions were dried with N<sub>2</sub>, and residues were then dissolved with assay buffer. We assayed samples in duplicate according to assay protocol, and separation of bound and free counts was achieved by the addition of dextran-coated charcoal. We processed known amounts through the entire procedure. We adjusted results for recovery and expressed results as ng/ml of plasma.

All samples were extracted once, and two assays were performed. Intra-assay variation was 3.6%, and interassay variation was 7.8%. The antiserum has 100% cross-reactivity with T, 45–50% with 5 $\alpha$ -DHT, 7.1% with 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 4.5% with 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 2.1% with androst-4-ene-3,17-dione, and less than 1.0% for other steroids. The lower limit of detection (2 SD at zero dose) was approximately 3 pg, according to assay specifications. The sensitivity of the assay based on actual recoveries and plasma volume was 1.1 ng/ml plasma.

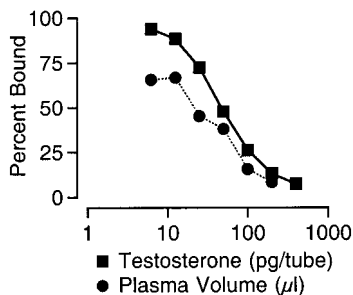


FIG. 1. Displacement curves of serially diluted male *Hyla cinerea* plasma (●) and testosterone standard (■) as measured with a radioimmunoassay kit (TRK-600, Amersham Pharmacia Biotech, Piscataway, NJ).

We validated the RIA kit for use with frog plasma in two ways. First, we determined that serial dilutions of pooled plasma samples from male *H. cinerea* paralleled standard dilutions (Fig. 1; test for homogeneity of slopes,  $t(9) = 0.06$ ,  $P = 0.9$ ). Second, we verified that there is no factor in frog plasma that interferes with the assay as follows. We measured testosterone levels in four samples (50  $\mu\text{l}$  each) from pooled plasma of castrated males or females to which we added either 0 or 800 pg of testosterone (2  $\text{pg}/\mu\text{l}$ ): castrated male, 79.9 pg; castrated male + T, 894.9 pg; female, 112.7 pg; female + T, 819.7 pg.

### Statistics

We used  $\chi^2$  tests to determine if hormone treatment influenced the likelihood of calling. We used analysis of variance (ANOVA) to determine effects of hormone manipulation and acoustic treatment (Hormone  $\times$  Stimulus) on spontaneous and evoked calling rates (averaged over the duration of the experiment) separately. We used repeated measures ANOVA to compare calling of the two defined time periods (spontaneous vs evoked) and to examine the diurnal pattern of calling (Hormone  $\times$  Stimulus  $\times$  Hour) in calling males only. We used Pearson's correlation to examine the relationship between androgen level and calling rate. Distribution of plasma androgen levels was non-normal as assessed by normality Q-Q plots, and these data violated the assumption of homogeneity of variance (as determined by Levene's test). Log transformation resulted in normally distributed data and improved, but did not fix, the homogeneity of variance problem. We also chose to log transform the calling rate data for correlation analyses in order to preserve potential relationships between call rate and androgen

level. Since one cannot log transform zero scores, a value of 1 was added to call rate scores prior to transformation. Castrated males were excluded from correlation analyses because they showed little to no variation in calling and androgen levels. Significance was determined at an  $\alpha$  level of 0.05 and all tests were two-tailed.

The research presented here was described in Animal Research Protocol No. 05962101C1 approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

## RESULTS

### Probability of Calling

Hormone treatment influenced the likelihood that a male would call at least once during the experiment ( $\chi^2(2) = 28.5$ ,  $P < 0.0001$ ; Fig. 2). Compared to intact males, castrated males were much less likely to call ( $\chi^2(1) = 38.8$ ,  $P < 0.0001$ ), whereas intact and T-implanted males were similarly likely to call ( $\chi^2(1) = 3.2$ ,  $P = 0.07$ ).

### Spontaneous Calling

Hormone treatment had strong effects on spontaneous calling ( $F(2, 65) = 21.1$ ,  $P < 0.001$ ; Fig. 3A). Post-hoc tests showed that intact males called more than castrated males ( $F(1, 48) = 15.9$ ,  $P < 0.001$ ), and T-implanted males called more than intact males ( $F(1, 45) = 12.8$ ,  $P = 0.001$ ). These differences cannot be attributed to differences in the proportion of calling males in each group, as analyses that excluded non-callers result in the same conclusions. The only excep-

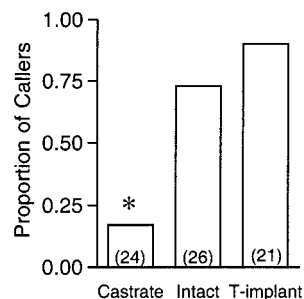
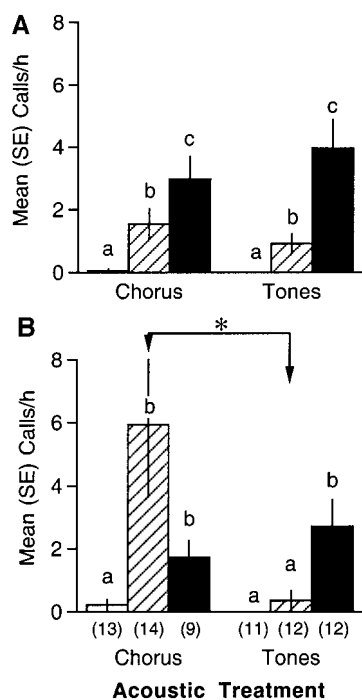


FIG. 2. The influence of hormone treatment on the proportion of males who called at least once during the experiment. Sample sizes are indicated in parentheses, and an asterisk indicates groups significantly different from the proportion of intact callers.



**FIG. 3.** The effect of hormone treatment and social signals on spontaneous (A) and stimulus-evoked (B) calling (mean  $\pm$  SE in all cases). Males were castrated (open bars), intact (hatched bars), or castrated and given a testosterone implant (filled bars). Sample sizes are indicated in parentheses (the same individuals are represented in A and B). Within each acoustic treatment, significant differences among hormone treatments are indicated by unique letters. Significant differences across acoustic treatments are indicated by an asterisk.

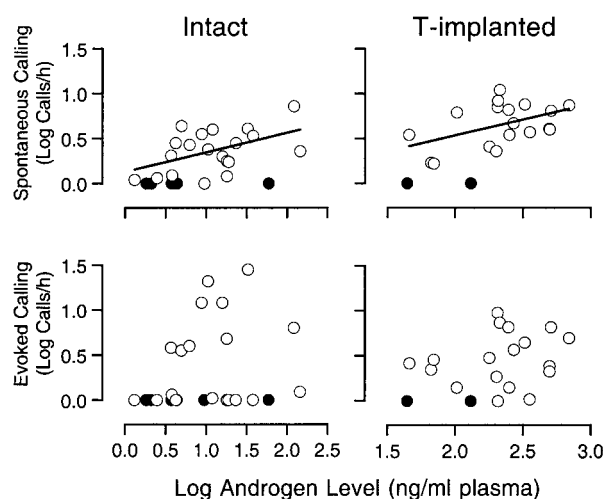
tion was that the difference between castrated and intact males did not reach significance ( $F(1, 21) = 3.83$ ,  $P = 0.064$ ) when only callers were considered. We also examined the influence of acoustic experience on spontaneous calling. Although listening to mating calls causes an increase in androgen levels (Burmeister and Wilczynski, 2000), and androgen levels increased spontaneous calling (above), reception of the chorus at night did not influence spontaneous calling.

The effect of androgen treatment on spontaneous calling was reflected in an overall positive correlation between spontaneous calling and plasma androgen levels ( $r = 0.66$ ,  $n = 46$ ,  $P < 0.001$ ; Fig. 4). This relationship was significant for intact males ( $r = 0.52$ ,  $n = 26$ ,  $P = 0.006$ ) and T-implanted males ( $r = 0.59$ ,  $n = 20$ ,  $P = 0.007$ ). This positive relationship also exists among callers only (overall,  $r = 0.66$ ,  $n = 37$ ,  $P < 0.001$ ; intact,  $r = 0.51$ ,  $n = 19$ ,  $P = 0.026$ ; T-implanted,  $r = 0.48$ ,  $n = 18$ ,  $P = 0.045$ ).

### Evoked Calling

Hormone manipulation and acoustic experience interacted in complex ways to influence stimulus-evoked calling (Hormone  $\times$  Stimulus,  $F(2, 65) = 4.48$ ,  $P = 0.015$ ; Fig. 3B). First, hormone treatment influenced the difference between chorus and tones in evoking calling: among intact males, those listening to the chorus called more than those listening to the tones ( $F(1, 24) = 5.11$ ,  $P = 0.033$ ), whereas, among T-implanted males, those listening to the chorus called at the same rate as tone-listening males ( $F(1, 19) = 0.8$ ,  $P = 0.4$ ). Second, hormone treatment had opposite effects on calling in tone-listening and chorus-listening males: in response to the tones, T-implanted males called more than did intact males ( $F(1, 22) = 6.6$ ,  $P = 0.018$ ), whereas in response to the chorus, intact males called more than T-implanted males, although mean differences did not reach significance ( $F(1, 21) = 2.1$ ,  $P = 0.16$ ), except when only callers were considered ( $F(1, 17) = 4.6$ ,  $P = 0.047$ ). These effects of hormones on evoked calling were not due to differences in the proportion of callers in each group, as statistical analyses that considered only callers supported the same conclusions.

Variation in androgen level did not explain variation in evoked calling (Fig. 4). When data from intact and T-implanted males are combined, the correlation between evoked calling and plasma androgen level was not significant ( $r = 0.26$ ,  $n = 46$ ,  $P = 0.078$ ). Furthermore, there was no relationship between an-



**FIG. 4.** The relationship between spontaneous or evoked calling and plasma androgen levels for intact and T-implanted males. Non-callers are indicated by filled symbols, and significant relationships are indicated by regression lines.

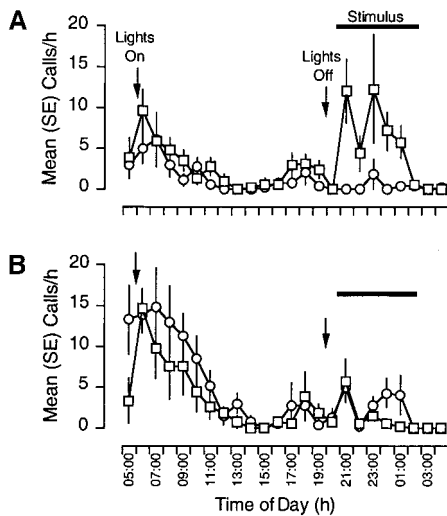


FIG. 5. Diurnal pattern of calling in intact (A) and testosterone-implanted (B) males receiving the chorus ( $\square$ ) or tones ( $\circ$ ) stimulus. Stimulus period is indicated by a bar and light cycle is indicated by arrows. Each point is a group mean ( $\pm$ SE) of individual mean calls for that hour. The sample size was 12 for each group, except for intact males receiving the chorus, which was 14.

drogen level and evoked calling among intact ( $r = 0.26$ ,  $n = 26$ ,  $P = 0.20$ ) or T-implanted males ( $r = 0.34$ ,  $n = 20$ ,  $P = 0.15$ ), nor was there any relationship among callers only (overall,  $r = 0.13$ ,  $n = 37$ ,  $P = 0.43$ ; intact,  $r = 0.17$ ,  $n = 19$ ,  $P = 0.48$ ; T-implanted,  $r = 0.19$ ,  $n = 18$ ,  $P = 0.48$ ).

### Spontaneous vs Evoked Calling

In the laboratory, green treefrogs call spontaneously at low rates during morning hours and show a specific increase in calling when presented with a mating chorus at night, but generally do not call when presented with tones (Burmeister and Wilczynski, 2000). We investigated the possibility that hormone treatment influenced this pattern of calling. Regardless of treatment, all groups showed the same overall pattern of calling (Hormone  $\times$  Stimulus  $\times$  Hour,  $F(12, 23) = 0.7$ ,  $P = 0.76$ ; Fig. 5). However, hormone treatment did influence the chorus-specific increase in call rate. Among intact males, there was a significant interaction between time period (spontaneous vs evoked) and acoustic stimulus ( $F(1, 24) = 4.45$ ,  $P = 0.046$ ), indicating that intact males increased calling in response to the chorus but not the tones. In contrast, the interaction was not significant for T-implanted males, and both chorus- and tone-listening males called more during the spontaneous period ( $F(1, 19) = 6.4$ ,  $P = 0.02$ ).

## DISCUSSION

We examined the influence of androgens on calling behavior in the presence and the absence of social signals. As in other frogs, calling depends on the presence of androgens, as castration abolished and T replacement maintained calling. Among intact and T-implanted males, the influence of hormone treatment on calling differed between spontaneous and evoked calling. There was a positive effect of androgen treatment on spontaneous calling and a positive correlation between plasma androgen level and spontaneous calling. The influence of androgens on evoked calling was more complex and interacted with acoustic treatment. First, hormone treatment influenced the chorus-specific increase in calling. Intact males called more in response to the chorus than the tones, whereas T-implanted males called at the same rate to both stimuli. In addition, in response to the chorus, T-implanted males called less than did intact males (among callers), in spite of the fact that they had higher androgen levels. Furthermore, variation in androgens did not explain variation in evoked calling.

Previous studies have demonstrated that calling depends on the presence of androgens (Wada and Gorbman, 1977; Wada *et al.*, 1976; Wetzel and Kelley, 1983) and that androgens are positively correlated with the motivation to call in free-living frogs (Burmeister *et al.*, 2001; Solís and Penna, 1997). However, androgen replacement following castration does not always restore or maintain calling at intact levels (Schmidt, 1966; Wetzel and Kelley, 1983), suggesting that the influence of gonads on calling is not mediated solely by androgens. In the present study, we demonstrated that androgens influenced the motivation to call spontaneously, but as in previous studies, androgen replacement failed to maintain evoked calling at intact levels. Furthermore, variation in androgen level did not correlate with variation in calling when males are socially stimulated (present study; Burmeister and Wilczynski, 2000).

Our results are consistent with steroid effects on courtship in other vertebrates, in that androgens are necessary for the expression of courtship, but absolute levels of androgens often do not explain individual variation in behavior. Individual variation in evoked calling is the result of many factors, which may include steroid binding globulins (Jennings, Moore, Knapp, Matthews, and Orchinik, 2000), steroid receptor sensitivity, and other aspects of physiology, such as stress and metabolism (Marler and Ryan, 1996). A good candidate for mediating evoked calling is the

neuropeptide vasotocin. Vasotocin influences the expression of social behavior in many vertebrates, and in frogs, vasotocin has potent effects on the motivation to call (Boyd, 1994a; Burmeister *et al.*, 2001; Chu, Marler, and Wilczynski, 1998; Marler, Chu, and Wilczynski, 1995; Penna, Capranica, and Somers, 1992; Propper and Dixon, 1997; Semsar, Klomberg, and Marler, 1998). Furthermore, vasotocin staining in the brain is androgen dependent (Boyd, 1994b) and differs between callers and noncallers in free-living cricket frogs (Marler, Boyd, and Wilczynski, 1999).

The inhibition of evoked calling by androgen implants is difficult to explain and may indicate that our treatment had pharmacological effects. T-implanted males differ from intact males in many ways, namely, T-implanted males have high unchanging plasma androgen levels. One possible reason for detecting a correlation between androgens and spontaneous but not evoked calling is the timing of plasma collection. We sacrificed males during the day, which corresponded in time with spontaneous calling but not with evoked calling. Diurnal fluctuations in steroids are common in vertebrates (Norris, 1997). It is possible that diurnal fluctuations in androgen level drive diurnal fluctuations in calling in frogs, and we would, therefore, find a positive correlation with evoked calling if we collected plasma during evoked calling. However, several lines of evidence fail to support this interpretation. First, spontaneous calling in T-implanted males demonstrated the same diurnal pattern as intact males, in spite of the fact that T levels do not fluctuate in implanted males. This does not support the interpretation that diurnal fluctuations in androgen level are required for diurnal fluctuations in calling. Second, if the lack of a correlation between evoked call rate and androgen level is the result of the timing of plasma collection, then we would still expect to elevate evoked calling when we elevated androgens. The failure of elevated androgens to increase evoked calling suggests that the observed lack of a correlation is real and that additional factors are important in evoked calling. Third, these results are consistent with findings in free-living treefrogs when plasma was collected at night during the chorus period: androgen concentrations were not correlated with how often males were observed calling, a gross measure of calling rate, although androgen level did influence the latency to resume calling after capture (Burmeister *et al.*, 2001). On the other hand, it is possible that androgen level did influence evoked calling, but that it changed aspects of evoked calling that we did not measure, such as the types of calls a male

produces (agonistic calls vs mating calls) or particular aspects of the mating call (e.g., call duration or dominant frequency). Clearly, further study needs to be done before the relationship between evoked calling and androgens is resolved.

In addition, high unchanging levels of plasma androgens in T-implanted males may interfere with other aspects of physiology. For example, castrated/T-implanted males may lack other hormones that are necessary for evoked calling. Due to negative feedback on the hypothalamus or pituitary, castrated/T-implanted males may lack gonadotropin releasing hormone (GnRH) or gonadotropins, which may be necessary for evoked calling (Schmidt, 1966; Wada and Gorbman, 1977; Wetzel and Kelley, 1983). Human chorionic gonadotropin (HCG) injections increased calling in intact as well as in castrated/androgen-implanted *Xenopus laevis*, suggesting that HCG effects on calling do not depend on the presence of gonads (Wetzel and Kelley, 1983; although see Palka and Gorbman, 1973). In fact, HCG injections were necessary to elevate calling in implanted males to the level of intact males. As in other vertebrates, androgens have a negative effect on gonadotropin secretion of anurans (McCreery and Licht, 1984; Pavgi and Licht, 1989). In our study, T implants presumably caused an inhibition of gonadotropin secretion through negative feedback on the brain and pituitary. If gonadotropins are important in calling in treefrogs, then a lack of gonadotropins may explain the reduced calling in implanted males.

Evidence also supports the possibility that high doses of steroids inhibited evoked calling through inhibition of GnRH. Previous studies implicate a role for GnRH in acoustically evoked calling. The auditory system has robust projections to GnRH control regions (Allison and Wilczynski, 1991; Neary, 1988; Wilczynski, Allison, and Marler, 1993), and social signals cause an increase in androgen level (Burmeister and Wilczynski, 2000), suggesting that listening to social signals increases GnRH secretion. Increased secretion of GnRH has implications beyond gonadotropin release, as evidenced by the extensive innervation of GnRH fibers throughout the brain (Jokura and Urano, 1986; Burmeister and Wilczynski, unpublished observation). GnRH itself may be involved in the neural control of sexual behavior in frogs, as it is in rats (Moss and McCann, 1973; Pfaff, 1973; Sakuma and Pfaff, 1983), birds (Maney, Richardson, and Wingfield, 1997), amphibians (Kelley, 1982; Moore, Miller, Spielvogel, Kubiak, and Folkers, 1982; Moore, Muske, and Propper, 1987), and lizards (Alderete, Tokarz, and

Crews, 1980). The implication is that reception of social signals causes an increase in GnRH secretion in the brain, where it may influence calling as a neuro-modulator. If such a scenario is true, suppression of a GnRH response to social signals by T implants would inhibit socially stimulated calling. Indeed, there is precedence for such an effect in other vertebrates: in female ring doves, low doses of estradiol promote sexual behavior, but high doses inhibit sex behavior through the inhibition of GnRH (Cheng, 1977). Although speculative at this point, a role for GnRH and gonadotropins in the neural control of calling clearly requires more attention.

In summary, we found that calling depends on androgens and that androgenic effects on calling depend on social context. Results from the current study are consistent with previous findings in which calling rate was not correlated with androgen level in socially stimulated males, but androgen level was higher in spontaneously calling males who were not socially stimulated (Burmeister and Wilczynski, 2000). Together, these data support an effect of androgens on the motivation to call spontaneously; however, when males are socially stimulated, androgens are necessary but insufficient to explain variation in calling. Finally, our data suggest that androgens alone are not responsible for courtship behavior in frogs and that other systems, such as GnRH and vasotocin, may also be involved.

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