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Behavioral and Hormonal Effects of Exogenous Vasotocin and Corticosterone in the Green Treefrog

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Vasotocin (AVT) promotes courtship in a wide range of vertebrates. However, this effect is not independent of steroid hormones. For example, androgens may work in concert with AVT and corticosterone (CORT) may work to oppose AVT action. In frogs, AVT promotes calling, and in some species, CORT inhibits calling. In addition, androgens are known to modulate AVT in the brain, and CORT may depress androgen secretion. Previous work in amphibians has suggested that AVT promotes courtship by overcoming a CORT-mediated stress response. Possible behavioral and hormonal interactions among AVT, CORT, and androgens were investigated in wild, free-living green treefrogs (*Hyla cinerea*). Saline, AVT, CORT, or a combination of AVT and CORT were administered to calling males, and several measures of spontaneous calling were evaluated for 1.5 h following injection. Plasma testosterone, dihydrotestosterone, and CORT were also measured. Saline-injected males had low CORT levels, and AVT and CORT injection elevated plasma CORT levels. AVT increased the likelihood of calling, but, in males who did call, AVT did not influence latency to call or how often they were observed calling. Very few saline-injected males resumed calling after injection, and therefore a CORT effect was only detectable in AVT-injected males. CORT inhibited calling in AVT-injected males only at the highest dose of CORT (40 μg); lower levels of CORT were unsuccessful at inhibiting AVT-induced calling. AVT appeared to have a specific effect on calling motivation. Further, the data suggest that disinhibition of a CORT response is not the primary mechanism by which AVT increases calling. In addition, CORT injection reduced endogenous androgen levels. Finally, endogenous androgens were negatively correlated with latency to begin calling, suggesting that they may have a positive effect on calling. These data indicate that AVT has positive effects on calling but provide only weak evidence that CORT inhibits courtship in this species.

Key Words: androgens; vasopressin; reproductive behavior; courtship; stress; amphibian; *Hyla cinerea.*

Hormones interact in complex ways to influence behavior. For example, sex steroids modulate peptide hormone systems, setting the stage on which peptide hormones act to promote reproductive behavior in a variety of species (Boyd and Moore, 1991; Coirini *et al.*, 1992; De Vries *et al.*, 1992; Moore *et al.*, 1992; Albers and Cooper, 1995). In addition, the stress hormone corticosterone (CORT) interacts with sex steroids by inhibiting androgen secretion (Moore and Zoeller, 1985; Moore *et al.*, 1991; Marler and Ryan, 1996) and reproductive behavior (Moore and Miller, 1984; Wingfield *et al.*, 1998). In newts, there is evidence that CORT interacts with the peptide hormone arginine vasotocin (AVT) by reversing the neurophysiological effects of AVT (Rose *et al.*, 1995). In frogs, AVT has potent effects on the motivation to call, which is critical to reproductive success (Ryan, 1985). In addition, data suggest an important role for AVT–steroid interactions in frogs (Penna *et al.*, 1992; Boyd, 1994b; Marler *et al.*, 1995); however, few such studies exist at this time.

A role for AVT in promoting the expression of reproductive behavior in amphibians is well established. In frogs, AVT appears to influence the motiva-

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tion to call, as AVT injection increases the likelihood of calling in several species, both in the laboratory and in the field (Penna *et al.,* 1992; Boyd, 1994a; Marler *et al.,* 1995; Propper and Dixon, 1997; Semsar *et al.,* 1998). In some cases, AVT injection influences particular aspects of the call, such as call duration (Klomberg and Marler, 2000) and dominant frequency (Marler *et al.,* 1995; Chu *et al.,* 1998), and influences the competitive ability of a calling frog (Semsar *et al.,* 1998). In addition, AVT in the brain differs between callers and noncalling satellite males (Marler *et al.,* 1999). In newts, AVT induces clasping in males (Moore and Miller, 1983) and appears to do so by influencing specific sensorimotor processes associated with reproductive behavior (Thompson and Moore, 2000). In frogs, the mechanism by which AVT influences calling is unknown.

AVT also interacts with steroids to affect reproductive behavior. For example, androgens were required for AVT-induced calling in the green treefrog (Penna *et al.,* 1992), a finding that is consistent with androgenic modulation of AVT in the amphibian brain (Boyd and Moore, 1991; Boyd, 1994b). Likewise, in the newt, sex steroids influence the behavioral response to AVT (Zoeller and Moore, 1982; Moore *et al.,* 1992) and AVT binding in the brain (Boyd and Moore, 1991). In the newt, AVT has been shown to interact with CORT at the level of the midbrain; AVT increases and CORT depresses neuronal responsiveness, and the two interact in complex ways when applied in combination (Rose *et al.,* 1995).

The role of CORT in reproductive behavior of frogs is less well understood. In some explosive breeders, increases in CORT and androgens are associated with the highly competitive period of breeding (Mendonça *et al.,* 1985; Orchinik *et al.,* 1988; Harvey *et al.,* 1997). Such changes may be associated with the stress of competition or with the metabolic demands of calling. CORT implants were effective at reducing calling and androgen levels in the seasonally breeding túngara frog (Marler and Ryan, 1996), a finding that is consistent with the inverse relationship between CORT and androgens in many species (Amario and Castellanos, 1984; Lance and Elsey, 1986; Moore *et al.,* 1991; Tsuchiya and Horii, 1995; Yajurvedi and Nijagal, 2000). In some frogs, capture and saline injection inhibits calling, whereas AVT restores the probability of calling to levels near those prior to the treatment (Marler *et al.,* 1995). Consequently, it was proposed that AVT may promote calling by overcoming the stress of capture and handling (Marler *et al.,* 1995). Although peripheral AVT is a stress hormone in amphibians and other vertebrates (Kloas and Hanke, 1990), vasopressin (the mammalian homologue of AVT) and CORT are known to interact at several levels in mammals: neural vasopressin inhibits CORT release (Kalsbeek *et al.,* 1992), and CORT can inhibit neurohypophysial release of vasopressin (Raff, 1987). While peripheral injections have been used invariably in frog studies to date, the assumption that peripheral AVT has access to the brain is reasonable (Moore and Miller, 1983), although untested, and raises the possibility of complex interactions between AVT and CORT.

These previous studies suggest that peptide–steroid interactions may be important in influencing male reproductive behavior in frogs. This possibility was investigated by examining the interaction of AVT and CORT treatment on spontaneous calling in male treefrogs (*Hyla cinerea*) under natural conditions. In addition, the possibility that endogenous androgens interact with AVT and CORT treatment to influence calling was investigated. To do this, several measures of calling were assessed following injection of saline, AVT, CORT, or a combination of AVT and CORT. Five hypotheses were addressed: (1) AVT injection promotes calling; (2) CORT injection inhibits calling; (3) AVT and CORT interact, such that the stimulatory effects of AVT are inhibited by CORT in a dose-dependent manner; (4) endogenous androgen levels influence the likelihood of calling and the likelihood that a male responds to AVT treatment; and (5) CORT depresses androgen levels.

METHODS

General Procedures

All subjects were naturally breeding male treefrogs at McKinney Falls State Park in central Texas. The experiment was performed between 2 July and 10 August 1999. Calling males were captured between 21:32 and 23:53 h, with the majority of subjects (76%) being captured before 22:30. Each male was immediately administered two 100-μl intraperitoneal injec-

tions of 0.9% saline (NaCl), 20 μg AVT, or one of three doses of CORT. As a result, each male received one of the following treatments: saline + saline; AVT + saline; 10 μg CORT + saline; 20 μg CORT + saline; AVT + 10 μg CORT; AVT + 20 μg CORT; AVT + 40 μg CORT. The highest dose of CORT (40 μg) was only administered in combination with AVT. There were 10 animals in each of the seven treatment groups. Following injection, males were placed in an acoustically transparent enclosure (wire mesh over a wooden frame; 27 × 27 × 41cm) near the chorus and their calling behavior was monitored for 1.5 h. Activity of the natural chorus generally extended until approximately 02:00, and the observation period of subjects always occurred during the natural chorusing period. We measured latency to begin calling (min) and assigned a calling score (number of 15-min periods in which a male was observed calling; maximum possible score was 6). At the end of the observation period, blood was collected by heart puncture from 60 animals. In order to reduce effects of acute handling on CORT levels, duration of heart puncture was limited to 3 min. Males were toe-clipped before release to prevent retesting. Blood was stored on ice until being centrifuged the same night as collection, and plasma was stored at −20° until radioimmunoassay.

Hormone Administration

For AVT treatment, we injected 20 μg AVT (Sigma Chemical Co., St. Louis, MO) dissolved in saline (see below), resulting in a dose of approximately 3 μg per gram body weight. Similar doses have previously been successful at evoking calling in frogs (Penna *et al.*, 1992; Boyd, 1994a; Chu *et al.*, 1998; Klomberg and Marler, 2000). Stress-induced CORT levels in frogs range from 14–75 mean ng/ml plasma (Juráni *et al.*, 1973; Zerani *et al.*, 1991; Coddington and Cree, 1995; Gobbetti and Zerani, 1996; Hopkins *et al.*, 1997); this variation appears to be more related to species differences than to the length or intensity of the stressor. Male treefrogs in our population weighed approximately 8 g. Based on previous studies in the newt (Moore and Miller, 1984; Moore and Zoeller, 1985), it was estimated that 10-μg CORT injections would result in approximately 60 ng/ml plasma levels, which is comparable to stress levels measured in other frogs. We chose to use 10, 20, and 40 μg CORT based on these data and preliminary behavioral effects in the treefrogs. CORT (Sigma Chemical Co.) was dissolved with a small volume of 90% ethanol before being brought to final concentration with 0.9% saline. All solutions, including saline and AVT (also dissolved in 0.9% saline) had a final ethanol concentration of 1%. Aliquots were stored at −20° and defrosted on the night of use.

Radioimmunoassay

Procedures and antisera are described in Burmeister and Wilczynski (2000). Briefly, steroids were extracted with organic solvents and then separated and partially purified on short celite:propylene glycol:ethylene glycol columns with a celite:water glycol trap. Steroid levels were measured by tritium-based competitive-binding radioimmunoassay. Prior to assays, ^{3}H-labeled steroid (approx. 2000 cpm) was added to each sample for recovery determinations. Water blanks and known amounts were processed through the entire procedure. Results were adjusted for recovery and expressed as ng/ml plasma. All samples were run in a single assay. Mean recoveries after extraction and chromatography were 73% for plasma testosterone (T), 67% for dihydrotestosterone (DHT), and 58% for CORT. Intraassay coefficients of variation were 4.5% (T), 6.5% (DHT), and 15.6% (CORT). The lower limit of detection for each assay, expressed as pg/tube, was 1.95 pg (T, DHT) and 3.9 pg (CORT).

Statistics

Analysis of variance (ANOVA) was used to analyze plasma hormone data. Plasma CORT data violated the assumption of homogeneity of variance (as determined by Levene's test), and log transformation corrected this problem. Consequently, log-transformed CORT data were used for statistical analyses. To analyze the effects of AVT and CORT on endogenous androgens, two categorical variables were created with as many levels as doses (AVT, 2 levels; CORT, 4 levels). We used multivariate analysis of variance (MANOVA) followed by ANOVA with these as independent variables in separate analyses. We could not statistically examine the interaction between AVT and CORT injection because we did not have all possible combinations of treatments (i.e., there was no 40 μg

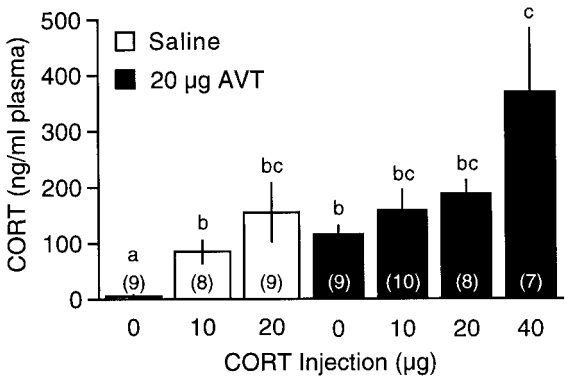

FIG. 1. Mean (±SE) plasma corticosterone levels 1.5 h following injection of saline, vasotocin, and/or corticosterone in the green treefrog. Statistically indistinguishable groups have one letter in common. Subject numbers are indicated in parentheses.

CORT + saline group). In order to determine if treatment influenced the likelihood of calling, we performed χ^2 analysis on the proportion of males calling in each group. The null hypothesis was defined as the proportion of calling males in the saline-only group. For the subset of males who called during the experiment, ANOVA was used to analyze calling score and latency to call. In addition, linear regression was used to assess the relationship between steroids and calling.

RESULTS

Hormonal Effects of Injection

CORT and AVT injection elevated plasma levels of CORT (ANOVA, $F_{6,52} = 38.39$, $P < 0.0001$; Fig. 1). Post hoc analyses indicate that all combinations of drug treatment elevated plasma CORT above that in saline-injected males. The group with the highest CORT injection (40 μg CORT + AVT) also differed from the AVT only group (AVT + saline) and the group receiving the lowest dose of CORT (10 μg CORT + saline). CORT levels in saline-injected males were low and similar to levels in nonstressed calling males (mean = 2.4 ng/ml plasma; Burmeister and Wilczynski, unpublished data).

Although there was no effect of AVT injection on androgens (T: ANOVA, $F_{1,58} = 0.002$, $P = 0.96$; DHT: ANOVA, $F_{1,58} = 0.013$, $P = 0.91$), CORT injection did reduce androgen levels (MANOVA: $F_{6,108} = 2.3$, $P = 0.04$; T: ANOVA, $F_{3,56} = 2.7$, $P = 0.05$; DHT: ANOVA, $F_{3,56} = 3.7$, $P = 0.02$, Fig. 2). We were unable to test for possible interactions between CORT and AVT injections; however, it is interesting to note that, although the AVT-only group had endogenous CORT levels as high as those of many of the CORT-injected males, they nonetheless had the highest mean androgen levels (38.7 ng/ml). Although CORT injection reduced mean androgen levels, there was no correlation between plasma CORT and androgens among individuals (T: $r = -0.18$, $n = 60$, $P = 0.16$; DHT: $r = -0.20$, $n = 60$, $P = 0.13$).

Behavioral Effects of Injection

Hormonal treatment had a significant effect on the likelihood that a male would call ($\chi^2 = 50$, $P <$

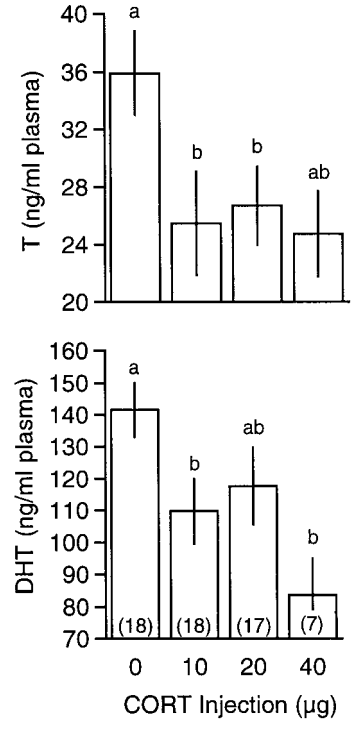

FIG. 2. Effect of corticosterone injection on mean (±SE) plasma testosterone (top) and dihydrotestosterone (bottom) 1.5 h after capture and treatment in the green treefrog. Approximately half of the males receiving 0, 10, or 20 μg CORT also received AVT, and all males receiving 40 μg CORT also received AVT; there was no effect of AVT treatment on androgen levels. Statistically indistinguishable groups have one letter in common. Subject numbers are indicated in parentheses.

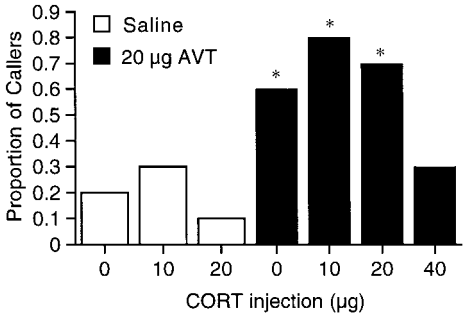

FIG. 3. Effect of capture and injection on the likelihood of calling in the green treefrog. *Significant difference from saline-only group. There were 10 animals in each of the seven treatment groups.

0.0001; Fig. 3). Males injected with CORT only (10 μg CORT + saline; 20 μg CORT + saline) were indistinguishable from control males and showed low probability of calling. AVT treatment increased the probability of calling when combined with saline or the two lower doses of CORT (10 or 20 μg). However, AVT failed to increase the likelihood of calling when combined with the 40-μg CORT injection. Among males who did call, AVT or CORT treatment did not influence calling score or latency to call.

Androgen levels did not differ between callers and noncallers, nor did androgen levels differ between those males who did and did not call in response to AVT treatment. However, among callers, there was a significant correlation between androgen levels and latency to begin calling (T: $r = -0.39$, $n = 27$, $P = 0.04$; DHT: $r = -0.42$, $n = 27$, $P = 0.03$; Fig. 4). Inspection of the data revealed that the variation in latency tended to be higher at low androgen levels. To test this, the residual of latency regressed on androgen levels (as shown Fig. 4, top) was calculated as a measure of variation. In fact, androgens were better at explaining residual latency than they were at explaining latency itself (T: $r = -0.45$, $n = 27$, $P = 0.02$; DHT: $r = -0.69$, $n = 27$, $P = 0.0001$; Fig. 4). The difference in r^2 between the regressions was quite large for DHT and represents an increase in explanatory power of 30%.

DISCUSSION

In our study, AVT influenced the probability to call and CORT inhibited AVT-induced calling at the highest CORT dose. In addition, endogenous androgens were correlated with latency to begin calling, but did not differ between callers and noncallers. Finally, exogenous CORT injection resulted in a depression of androgens, although there was no overall correlation between the level of CORT and T or DHT.

These data contribute to a growing body of evidence that AVT increases the motivation to call in frogs (Boyd, 1994a; Marler *et al.*, 1995, 1999; Propper and Dixon, 1997; Semsar *et al.*, 1998). AVT injection increased the likelihood of calling in spite of elevated CORT levels, suggesting that the AVT effect is quite potent. However, among males who did call, AVT did not influence the latency to call or calling score, suggesting that, in this species, AVT does not enhance spontaneous calling beyond the likelihood to call. Previous studies have found an effect of AVT on latency to call (Boyd, 1994a; Chu *et al.*, 1998). This difference may be due to the fact that these previous studies measured evoked calling in response to conspecific calls, whereas the dependent measure in the current study was latency to begin spontaneous calling after capture. In any case, the difference in the findings does not suggest conflicting interpretation, as decreas-

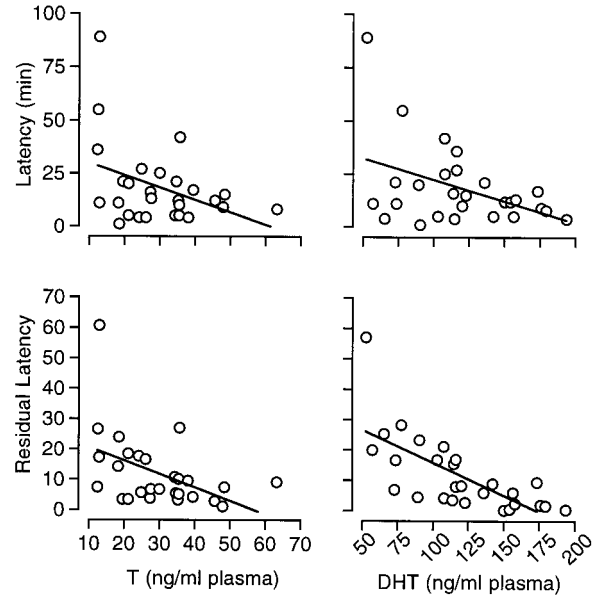

FIG. 4. Relationship between endogenous androgens and latency to begin calling. The correlations between latency to call and testosterone (top left) and dihydrotestosterone (top right). The correlation between residual latency (calculated from regression lines at top) and androgens (bottom).

ing latency to call and increasing probability to call are both indications of an increase in the motivation to call. Instead, these results may suggest that the phenotype of AVT-induced calling may be influenced by social context (Chu *et al.*, 1998; Klomberg and Marler, 2000) or simply species differences. Although species differences are certain to occur, the similarity of AVT action on frog calling in a variety of species is noteworthy.

The present data are more equivocal on the question of whether CORT affects calling. CORT-injected males called at the same rate as did saline-injected males, in spite of very different CORT levels. However, because capture and injection inhibited calling, this potentially created a "floor effect" against which we were unable to demonstrate a direct inhibitory effect of CORT. Nonetheless, AVT-induced calling provided an opportunity for inferring a CORT effect. In AVT-injected males, CORT failed to inhibit calling at the two lowest doses (10 and 20 μg). Only at the highest dose of CORT (40 μg) was CORT able to inhibit AVT-induced calling. Previous studies found that acutely stressed frogs have CORT levels ranging from 11 to 56 ng/ml plasma (Juráni *et al.*, 1973; Coddington and Cree, 1995), and chronically stressed frogs were found to have CORT levels from 14 to 75 ng/ml plasma (Zerani *et al.*, 1991; Gobbetti and Zerani, 1996; Hopkins *et al.*, 1997). In our study, AVT induced mean endogenous CORT secretion of 114 ng/ml plasma. Although stress-induced CORT levels in green treefrogs are unknown, these data suggest that the highest CORT dose (40 μg) resulted in plasma levels (mean of 363 ng/ml) that were outside the range of naturally occurring stress levels. Therefore, our data suggest that physiological levels of CORT were unsuccessful at inhibiting calling in the treefrog. However, this conclusion must remain tentative until a better understanding of stress-induced CORT levels are known in this species. The existing literature on the effects of CORT and stress on courtship behavior in amphibians is sparse. Although elevated CORT levels are positively associated with breeding in some explosively breeding frogs (Mendonça *et al.*, 1985; Orchinik *et al.*, 1988; Harvey *et al.*, 1997), chronic (Marler and Ryan, 1996) and acute (Moore and Miller, 1984) administration of CORT inhibits courtship in some seasonally breeding amphibians. Furthermore, administration of exogenous CORT does not always replicate the effects of stress on sexual behavior (Retana-Marquez *et al.*, 1998). Clearly, a more detailed investigation of the role of stress and reproduction in this species is necessary before this issue is resolved.

A previous study suggested that AVT may enhance calling by overcoming the stress of capture and handling (Marler *et al.*, 1995), an action that is consistent with AVT–CORT interactions in the newt (Rose *et al.*, 1995) and the effects of neural vasopressin on CORT and stress in mammals (Kalsbeek *et al.*, 1992; Buwalda *et al.*, 1993). However, previous studies did not include measures of endogenous CORT levels in response to saline or AVT injection. In our study, AVT treatment induced a substantial increase in endogenous CORT, an action consistent with its role as a peripheral stress hormone (Kloas and Hanke, 1990), whereas saline-injected males had very low levels of CORT. Similar CORT responses likely occurred in these previous studies. In our study, capture and injection only required a few minutes before the animal was placed in an enclosure, and the same was likely true for earlier studies (Marler *et al.*, 1995; Chu *et al.*, 1998; Semsar *et al.*, 1998). In frogs, significant elevation of CORT often requires more severe stressors, such as immobilization (Juráni *et al.*, 1973; Licht *et al.*, 1983) or longer-term capture (e.g., 24–72 h; Zerani *et al.*, 1991; Coddington and Cree, 1995; Gobbetti and Zerani, 1996). Thus, the AVT effects on calling reported by us and previous authors would not likely be explained by AVT overcoming a CORT response to the stress of capture and injection. Although CORT did not rise in response to capture and handling, our procedures did inhibit calling, suggesting that the animals may be stressed by these procedures.

Rather, our hormone data suggest that the AVT effect on calling is independent of a CORT-mediated stress response. Whereas AVT was able to induce calling in CORT-treated males, perhaps overriding undetectable inhibitory effects of CORT, the fact that saline-injected males did not call in spite of their low CORT suggests that disinhibition of a CORT response is not the primary mechanism by which AVT increases calling. Instead, the data suggest a specific effect on the motivation to call. The location and distribution of immunoreactive AVT cells and fibers in the frog brain is consistent with this interpretation (Boyd *et al.*, 1992). AVT cells or fibers are found in important regions of the descending vocal control pathway: striatum, ante-

rior preoptic area, and the pretrigeminal nucleus. In addition, AVT cells and fibers are found in the amygdala and nucleus accumbens (Boyd *et al.*, 1992; Marler *et al.*, 1999), brain regions implicated in sexual motivation in many vertebrates. Alternatively, there may be other mediators of short-term stress, such as catecholamines, that may be responsible for the inhibition of calling in saline-injected males, and AVT may be acting by opposing this system.

Several lines of evidence support a role for androgens in modulating AVT effects in frogs, although we were unable to provide support for a behavioral correlate of androgen–AVT interactions in the current study. There are several possible reasons for our negative finding. We may have reduced necessary variation by including only callers in the experiment and by using only one dose of AVT. A previous laboratory study found that androgens were required for AVT-induced calling in the green treefrog (Penna *et al.*, 1992). Whereas castration and hormone replacement indicate that AVT production and binding are androgen dependent (Boyd and Moore, 1991; Boyd, 1994b), there may not be differential modulation of AVT by androgens within the normal range of variation within calling males.

Although androgens did not differ between males that resumed calling and those that did not, there was a relationship between androgens and latency to begin calling, which suggests that natural variation in androgen levels does influence the motivation to call. Interestingly, androgen levels explained the *variation* in latency to call as well as or better than latency itself–increasing androgens reduced the variance associated with latency to call. This finding is consistent with the concept that steroid hormones do not induce behaviors, but rather influence behavioral tendencies. Several laboratory studies have demonstrated that calling depends on the presence of androgens (Wetzel and Kelley, 1983), but the role of endogenous androgens in calling is less well documented. Androgens were found to be lower in calling versus noncalling toads (Mendonça *et al.*, 1985; Orchinik *et al.*, 1988), but higher in calling coqui (Townsend and Moger, 1987) and túngara frogs (Marler and Ryan, 1996). Endogenous androgens are positively correlated with evoked calling in *Batrachyla taeniata* (Solís and Penna, 1997) and were higher in spontaneously calling green treefrogs in the lab (Burmeister and Wilczynski, 2000). Although some exceptions remain to be resolved, on the whole the data suggest that natural variation in endogenous androgens influences variation in calling.

In many seasonally breeding animals, elevation of CORT results in depression of androgen levels (Moore and Zoeller, 1985; Lance and Elsey, 1986; Moore *et al.*, 1991; Tsuchiya and Horii, 1995; Marler and Ryan, 1996). In frogs, androgen levels decline following capture (Licht *et al.*, 1983; Zerani *et al.*, 1991), although CORT may not rise until after androgens decrease (Zerani *et al.*, 1991). In our study, exogenous CORT resulted in a depression of androgen levels. However, it is interesting to note that AVT-induced endogenous elevation of CORT did not result in a decline in androgens. This could be due to a compensatory effect of AVT on androgen secretion directly or through some other factor. Certainly, AVT action on the adrenals likely has multiple effects in addition to CORT production.

In conclusion, AVT injection and endogenous androgens promote calling in the green treefrog, but there was no behavioral evidence for an AVT–androgen interaction. AVT action appeared to be a specific effect on the motivation to call, and did not act through disinhibition of a CORT-mediated stress response. In addition, although CORT was unable to inhibit calling at physiological levels, it depressed endogenous androgen levels. Finally, the data suggest that AVT may be able to counteract a depression of androgens by endogenous CORT.

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