



# The neuropeptide arginine vasotocin alters male call characteristics involved in social interactions in the grey treefrog, *Hyla versicolor*

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We investigated the effects of different doses (0, 2.5, 25 and 250 µg) of the neuropeptide arginine vasotocin (AVT) on the calling characteristics of the grey treefrog in a chorus in its natural habitat. AVT changed some call characteristics known to influence social behaviour in grey treefrogs. It increased call duration and number of pulses in a call, but not dominant frequency, call rate or pulse effort. Saline injections and handling did not produce significant changes in any of the call characteristics. In addition, individual animals injected with AVT only rarely produced call characteristics that were outside of the range found in the preinjection measurements, suggesting that AVT does not cause abnormal calling behaviour. Other researchers have demonstrated that longer calls with more pulses are produced by males when chorus densities increase, and females display a strong preference for longer calls with more pulses. This suggests that the changes induced by AVT injections may have functional consequences in social interactions. We previously demonstrated that AVT-injected males (25 µg AVT) displaced resident males from calling sites through changes in calling behaviour under natural field conditions. Our results indicate that changes in call duration and pulse number could contribute to the unmanipulated resident male's behaviour towards the AVT-injected intruder, perhaps because the calls are more attractive to females or because the calls are perceived as more aggressive.

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Arginine vasotocin (AVT) and the mammalian homologue arginine vasopressin (AVP) are neuropeptides that influence social behaviour in a number of taxa, including amphibians (summary by Boyd 1997) and mammals (e.g. Carter et al. 1995; Young et al. 1998). In anurans specifically, AVT increases the motivation to call in several species: grey treefrogs (Semsar et al. 1998), cricket frogs, *Acris crepitans* (Marler et al. 1995a; Chu et al. 1998), bullfrogs, *Rana catesbeiana* (Boyd 1994), green treefrogs, *Hyla cinerea* (Penna et al. 1992) and great plains toads, *Bufo cognatus* (Propper & Dixon 1997). Brain AVT immunoreactive levels are also linked to calling behaviour in male cricket frogs. Satellite males do not call themselves, but parasitize calling males by intercepting approaching females. Males expressing these two behavioural strategies immediately prior to fixation of the brain also have different brain AVT-immunoreactive levels (Marler et al. 1999). Calling males have higher levels of AVT-immunoreactive staining than satellite males in the nucleus accumbens, an area of the brain associated with

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motivation in other species (e.g. Packard et al. 1997). It is therefore a reasonable hypothesis that calling males release more AVT than satellite males and that AVT induces calling behaviour.

Arginine vasotocin not only influences the production of acoustic communication, but it also has an effect on the receiver of the altered communication signals (Semsar et al. 1998). As in many species, a resident male *H. versicolor* normally has an advantage in a male–male encounter. Once a male is in possession of a calling site, other males appear to be less likely to displace him (Fellers 1979). Intruders compete for calling sites through a combination of advertisement calls, aggressive calls and wrestling. AVT injections, however, cause a departure from typical interactions of male grey treefrogs. Males given AVT injections are more likely to displace resident males from a calling site, and this behaviour is probably mediated through acoustic communication. We do not, however, know why males give up a calling site when presented with the calls of an AVT-injected male. Understanding the negation of the 'resident advantage' via an increase in AVT as reported in Semsar et al. (1998) is the motivation behind the current study.

Male–male aggressive encounters in grey treefrogs appear to be mediated primarily through call properties alone because males rarely come into direct physical contact with one another (Schwartz 1987), yet little is known about the call characteristics that influence the resolution of an aggressive encounter. Males presented with conspecific calls increase both the call duration and pulse number while maintaining their call effort (call duration  $\times$  call rate; a measure similar to pulse effort) and decreasing their advertisement call rate (Wells & Taigen 1986). However, it is not known what function these changes serve. Male *H. versicolor* also use short aggressive calls, which differ acoustically from advertisement calls, when aggressive encounters escalate between males (Schwartz 1987). However, males can displace each other without the use of aggressive calls or physical aggression (Semsar et al. 1998).

In contrast to aggressive calls, more is known about how the properties of the advertisement calls influence female choice. Some properties used by females to assess a male's competitive status include call effort and pulse effort, call rate, call duration, pulse number and dominant frequency (Ryan 1980; Wells & Taigen 1986; Klump & Gerhardt 1987; Gerhardt 1991, 1992; Sullivan & Hinshaw 1992; Runkle et al. 1994; Ryan et al. 1995; Gerhardt et al. 1996). Call rate and pulse effort may indicate health or vigour because calling is the most energetically expensive activity for a male (Runkle et al. 1994). Females prefer a greater call rate, possibly because this indicates a male that is more successful in acquiring food or resisting disease. Interestingly, Welch et al. (1998) showed that males producing a longer call duration sire young that develop faster, which may confer a fitness benefit.

To examine how AVT negates the resident advantage by changing the properties of calls, we recorded male grey treefrogs in the field prior to and after an injection of one of three doses of AVT in saline or saline alone. We then examined several call properties: call duration, pulse number, pulse effort, dominant frequency and call rate.

## METHODS

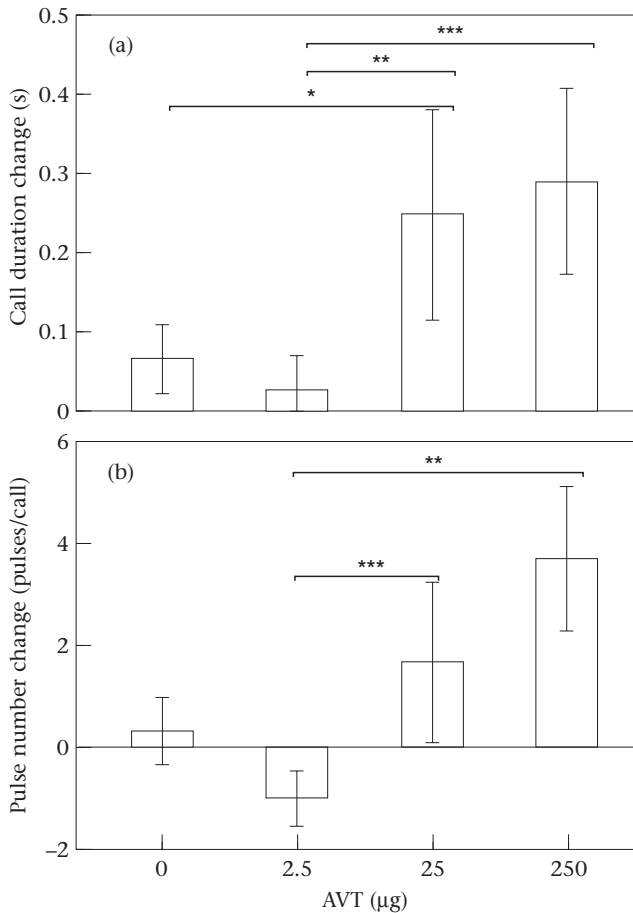
We conducted the study in the wetlands of south-central Wisconsin during summer 1997 and 1998, from late May to mid-August. Experiments were carried out between 2130 and 0100 hours, which is the peak calling period of *H. versicolor*. Once we located a calling animal with the aid of a flashlight, we made a 5 min recording of the male's vocalizations with a Sennheiser directional microphone (model ME67 with a K6 power unit) attached to a Marantz portable recorder (model PMD430). After the call was recorded, the frog was captured by hand.

We injected the captured male intraperitoneally with one of three randomly assigned doses of AVT (Sigma Chemical Co., St Louis, Missouri) suspended in 25  $\mu$ l of saline or the saline alone ( $N=7$ ): low: 2.5  $\mu$ g ( $N=12$ ); medium: 25  $\mu$ g ( $N=8$ ); and high: 250  $\mu$ g ( $N=8$ ). The medium and low doses were similar to those used in other studies (grey treefrogs: Semsar et al. 1998, 25  $\mu$ g AVT;

cricket frogs: Chu et al. 1998, 10  $\mu$ g/g body weight; Marler et al. 1995a, 10  $\mu$ g/g body weight; bullfrogs: Boyd 1994, 1.3  $\mu$ g/g body weight; green treefrogs: Penna et al. 1992, 25  $\mu$ g; great plains toads: Propper & Dixon 1997, 1–100  $\mu$ g/100 g body weight). After the injection we recorded the animal's leg length and snout-to-cloaca length. Next we marked the male by tying a cotton thread around his waist to which a number was attached (Morris 1989). The waist band did not appear to hinder the animal's movement or its ability to breed, as multiple males with waist bands were observed on successive nights, some in amplexus. After the male was marked, he was placed in a 1 litre plastic bag for 30 min to allow the injection to take effect (Marler et al. 1995a; Chu et al. 1998; Semsar et al. 1998). During this time we recorded the air and water temperatures at the male's calling site. Once 30 min had elapsed we returned the male to his original position (prior to capture) and made a second 5 min recording of his vocalizations as soon as the male began calling. All males called within 15 min after being returned to their calling sites. The four treatment groups did not have equal numbers of subjects because some frogs left the area before we were able to make a second recording. No other animals within 1 m of any of the subject animals were observed during either preinjection or postinjection recordings.

We conducted sound analysis with the computer program Cool Edits<sup>®</sup> provided by Syntrium Software. Complete recordings were digitized at 32 kHz with 16 bit resolution. This sampling rate is more than twice the greatest perceivable frequency (less than 14 000 Hz) so antialiasing filters were not necessary. We chose 20 sequential calls from approximately the middle of the recording. We defined the beginning of a call as the first zero crossing in the waveform of the sample. The end of a call was defined as the last zero crossing in the waveform of the sample. We analysed each of the 20 calls individually to determine the following characteristics: call duration (s), pulse effort (pulses/min; from the beginning of the first call to the end of the 20th call), pulse number (pulses/call) and dominant frequency (Hz). Dominant frequency was measured in the central three-quarters of a given call to reduce contamination of the frequencies by the overlapping calls of nearby frogs. Call rate was measured by recording the number of times males called during the 5 min recording (calls/min). Values corresponding to the 20 selected calls were analysed statistically.

To test for handling effects we compared pre- and post-injection call characteristics in the saline control group using paired *t* tests. We conducted an analysis of variance (ANOVA) on the data for each call characteristic to determine the effects of the manipulations between groups. The independent variable for all ANOVA analyses was injection type (four levels). We analysed postinjection call duration, pulse number, dominant frequency, call rate and pulse effort using the corresponding preinjection condition as a covariate. We used leg length and snout-to-cloaca length as additional covariates for dominant frequency because size is known to influence dominant frequency (Gerhardt



**Figure 1.** The change in (a) call duration and (b) pulse number from pre- to post-injection for males given saline, 2.5 (low), 25 (medium) or 250 µg (high) AVT. \*NS ( $P=0.051$ ), \*\* $P<0.05$ , \*\*\* $P<0.02$ .

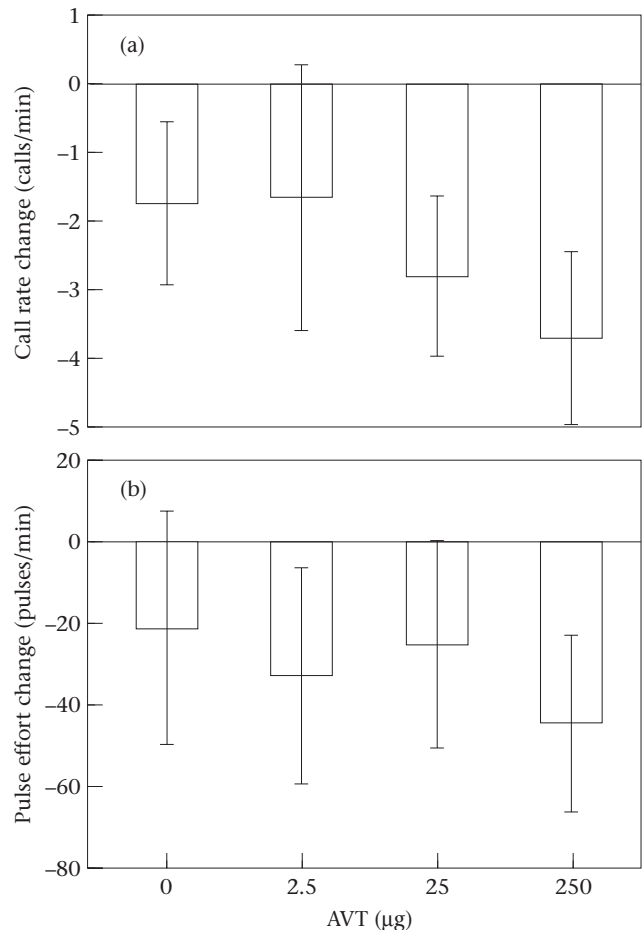
1994). We compared means using a Tukey honest significant difference (HSD) for unequal  $N$  for variables with significant results. All statistical results are reported as means  $\pm$  SE.

### Ethical Note

The social interactions did not result in any physical contact and therefore no animals were injured.

### RESULTS

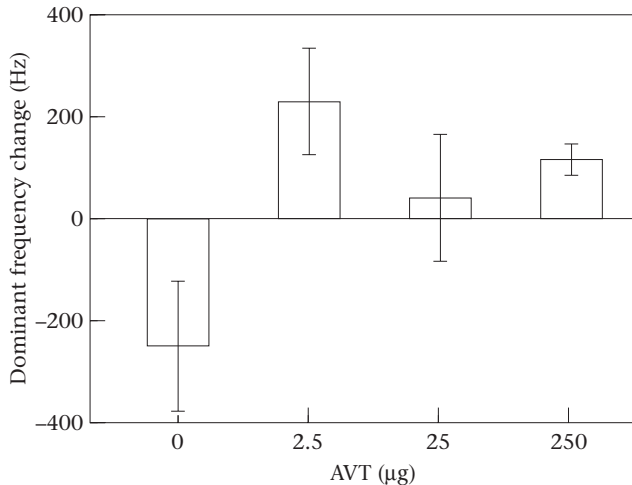
The handling manipulation alone did not cause a change in call characteristics as there were no significant differences between the pre- and post-injection call characteristics in the saline control group: call duration ( $t_6 = -1.55$ ,  $P=0.17$ ); pulse number ( $t_6 = -0.47$ ,  $P=0.66$ ); dominant frequency ( $t_6 = 1.94$ ,  $P=0.10$ ; note the non-significant tendency); call rate ( $t_6 = 1.46$ ,  $P=0.19$ ); pulse effort ( $t_6 = 0.73$ ,  $P=0.50$ ). In the treatment group comparisons, AVT-injected males displayed a significant increase in two of the call characteristics: call duration ( $F_{3,30} = 3.29$ ,  $P=0.03$ ; Fig. 1) and pulse number ( $F_{3,30} = 4.74$ ,  $P=0.01$ ; Fig. 1). Tukey HSD post hoc tests revealed a significant



**Figure 2.** The change in (a) call rate and (b) pulse effort from pre- to post-injection for males given saline, 2.5 (low), 25 (medium) or 250 µg (high) AVT.

difference in call duration between the low and medium AVT doses ( $P=0.01$ ), between the low and high doses ( $P=0.05$ ) and a nonsignificant tendency between the saline and medium doses ( $P=0.051$ ). Other pairwise post hoc comparisons for call duration did not approach a level of significance, suggesting that there may be a threshold effect between 2.5 and 25 µg. We also found a significant increase in pulse number between the low and medium doses ( $P=0.019$ ), and between the low and high doses ( $P=0.021$ ). Other post hoc  $P$  values for pulse number did not approach a level of significance (all  $P$ s NS). The other call characteristics did not show significant differences between the pre- and post-injection conditions: call rate ( $F_{3,30} = 1.98$ ,  $P=0.14$ ; Fig. 2); pulse effort ( $F_{3,30} = 0.29$ ,  $P=0.83$ ; Fig. 2); dominant frequency (leg length as a covariate:  $F_{3,29} = 1.15$ ,  $P=0.35$ ; snout-to-cloaca length as a covariate:  $F_{3,29} = 1.14$ ,  $P=0.35$ ; Fig. 3).

We examined the postinjection values for each call characteristic in every animal in relation to the unmanipulated preinjection limits (collapsed across all four treatment levels) to determine whether postinjection values were beyond the range of the unmanipulated calls. Animals that did have post-injection values outside of the observed preinjection limits were not representative of



**Figure 3.** The change in dominant frequency from pre- to post-injection for males given saline, 2.5 (low), 25 (medium) or 250  $\mu$ g (high) AVT.

any one treatment group (Table 1). We did not find significant differences in air temperature ( $F_{3,30}=1.30$ ,  $P=0.29$ ), water temperature ( $F_{3,30}=1.70$ ,  $P=0.19$ ), leg lengths ( $F_{3,31}=0.13$ ,  $P=0.94$ ) or snout-to-cloaca lengths ( $F_{3,31}=0.11$ ,  $P=0.96$ ) between the groups.

## DISCUSSION

The grey treefrog responded to AVT injections by changing call characteristics that may play a significant role in communication. Call duration and pulse number, but not call rate, pulse effort or dominant frequency, significantly increased after injections of a range of AVT doses. These changes did not appear to be a function of the stress of injecting and handling because similar changes were not observed in the saline-injected controls. In addition, the calls produced were generally within the range of natural variation. Our results indicate that peripheral injections of AVT between 2.5 and 250  $\mu$ g (with a possible threshold effect between 2.5 and 25  $\mu$ g) caused unidirectional changes in both call duration and pulse number. These changes in dynamic call characteristics (Gerhardt 1991) could have social consequences. Other studies have demonstrated that males presented with conspecific calls increase call duration and pulse number and that females prefer calls that are longer and have more pulses (Wells & Taigen 1986; Klump & Gerhardt 1987; Sullivan & Hinshaw 1992; Gerhardt et al. 1996). Thus these call characteristics change in aggressive contexts and have important consequences for female choice, suggesting that information in AVT-induced calls could influence a wide range of social interactions.

Our results differ from a recently published paper examining the effects of AVT on calling behaviour in a different population of *H. versicolor*. Tito et al. (1999) found no effect of AVT on call duration or pulse number, as we did, but did find a significant decrease in call rate and slight increase in dominant frequency when comparing pre- ( $N=5$ ) and postinjection ( $N=7$ ) recordings of

males receiving 100  $\mu$ g injections of AVT. The most significant difference between the two studies is that saline-injected males gave post-injection calls in our study, but not in the study by Tito et al. (1999). Stress or other factors could cause this variation, but unfortunately the lack of postinjection recordings for saline-injected males precludes an analysis of stress effects on call characteristics. The methodology used by Tito et al. (1999) differed from our study in a number of ways that could influence stress or result in different social conditions such as recording males from Styrofoam containers in the field as opposed to allowing males to be free ranging. The differences between the two studies could potentially influence stress effects on calling behaviour, as has been documented for other anuran species (e.g. Marler et al. 1995a).

Our current results help to interpret our previous study demonstrating that AVT injections can functionally change behaviour in the grey treefrog (Semsar et al. 1998). Resident males retreated from their calling sites in response to the altered behaviour of the AVT-injected males. Several hypotheses could explain this behavioural change. One hypothesis is that AVT-induced calls are more aggressive thereby inducing the residents to retreat. In mammals there is a positive association between arginine vasopressin and aggressive/dominance behaviours (e.g. Winslow et al. 1993; Ferris et al. 1997; Bester-Meredith et al. 1999). As mentioned earlier, male *H. versicolor* presented with conspecific calls increase call duration and pulse number, suggesting that this increase indicates a more aggressive advertisement call (alternatively males may increase efforts to attract females when other males are nearby; Wells & Taigen 1986). This hypothesis is neither supported nor refuted because while AVT causes similar changes, the significance (i.e. aggression) of the call changes found in more dense choruses is unknown. AVT injections in *A. crepitans* also does not cause increased aggression during simulated encounters (Chu et al. 1998). Our data, however, suggest that while AVT-injected males displaced residents in the previous study without displaying physical aggression or aggressive calls, males could express aggression via changes in advertisement calls.

The resident males' retreat in response to AVT-injected males could also be explained by the hypothesis that AVT-induced calls were more attractive (Semsar et al. 1998). The results are consistent with this hypothesis because AVT-induced calls had longer durations with more pulses, characteristics preferred by females (e.g. Sullivan & Hinshaw 1992; Gerhardt et al. 1996). In addition male frogs with longer call durations and greater pulse numbers sire faster developing young (Welch et al. 1998). Although the effects of AVT on calls of *H. versicolor* contrast with its effects on the calls of *A. crepitans*, the changes are consistent with differences in female preferences between the two species. In males, AVT increased pulse number per call in *H. versicolor*, yet it decreased pulse number in *A. crepitans* (Marler et al. 1995a). AVT may therefore increase the attractiveness of both species' calls because females prefer higher pulse numbers in *H. versicolor* (Gerhardt et al. 1996), but lower pulse numbers in *A. crepitans* (Wagner 1991; but see Perrill & Lower

**Table 1.** Preinjection range of call properties and postinjection values outside of the preinjection range

Property	Preinjection range (N=35)	Preinjection mean±SE	Treatment	Postinjection values outside of preinjection range
Call duration (s)	0.37–1.54	0.77±0.05	Saline Low AVT Medium AVT High AVT	None 0.34 1.68 None
Pulse number	7.90–25.95	15.36±0.74	Saline Low AVT Medium AVT High AVT	None None None None
Call effort (pulses/min)	67.55–299.98	197.38±10.78	Saline Low AVT Medium AVT High AVT	None 61.56 None None
Call rate (calls/min)	6.62–23.83	12.36±0.68	Saline Low AVT Medium AVT High AVT	6.28 3.75 3.57 1.57
Dominant frequency (Hz)	1729.30–3077.65	2159.64±45.17	Saline Low AVT Medium AVT High AVT	None 1709.06 None None

Postinjection values outside of the preinjection range were rare (never more than one for a given call property in a given treatment group), and were close to the edge of the range for the given property. No animal was outside of the preinjection range for more than one property.

1994). Resident males may retreat from their calling sites in response to AVT-injected intruders either to avoid AVT-injected males temporally by abandoning calling to save energy for use on another night when the (more attractive) AVT-injected male is not present, or to avoid competition spatially by moving to a more distant part of the chorus.

A third hypothesis is that AVT calls are outside of *H. versicolor's* normal range. Residents may perceive this proposed abnormality and retreat from the injected male. In the present study, however, only a few animals had AVT-induced call properties outside of the preinjection range. In addition, call properties were similar to those in other studies (e.g. Bogart & Jaslow 1979; Runkle et al. 1994). Our data, therefore, do not support the hypothesis that AVT-induced calls were abnormal.

Clearly more studies are required to test and develop each hypothesis. If further studies support the female preference hypothesis or the male aggression hypothesis, then why do males not always produce longer calls with more pulses, as caused by increased AVT? It is possible that these AVT-induced calls may be more energetically costly. Calling behaviour is the most energetically expensive activity for *H. versicolor*, with oxygen consumption reaching 30 times resting metabolic rate (Wells & Taigen 1986; Runkle et al. 1994). Males producing long calls have a lower respiratory quotient than those making short calls and probably catabolize a higher percentage of lipids (Grafe 1997). Energetic costs of a behaviour could lead to selection pressures decreasing the expression of

that behaviour, as in the lizard *Sceloporus jarrovi* (e.g. Marler & Moore 1988; Marler et al. 1995b). In *H. versicolor* this may lead to a decrease in the use (or level) of AVT as a mechanism for influencing calling behaviour.

In conclusion, there was a positive effect of AVT on call duration and pulse number. Arginine vasotocin caused call changes that may influence the attractiveness or aggressive nature of a frog's call. Further studies examining male aggression and female choice using playbacks of calls are needed to separate the aggressive and attractive aspects of the calls. Comparisons among other anuran species will be useful for testing whether AVT acts to increase attractiveness of male calls even when the traits preferred by females differ among species.

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