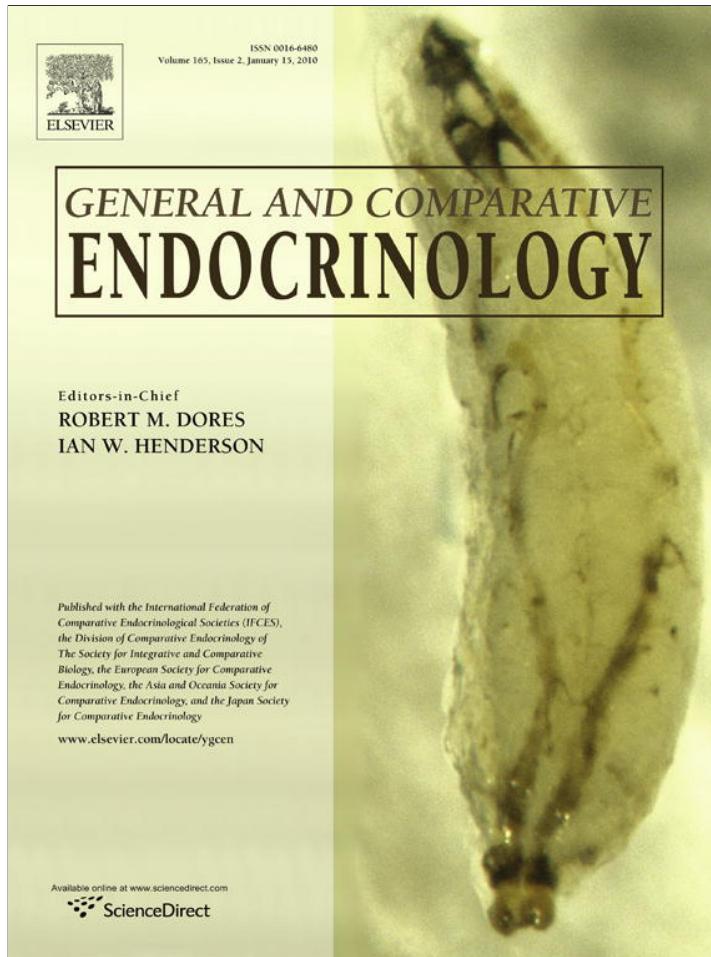


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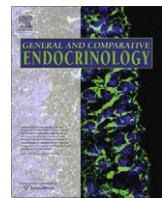
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Treatment with arginine vasotocin alters mating calls and decreases call attractiveness in male túngara frogs

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ABSTRACT

The peptide hormone arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin modulate a variety of social behaviors in vertebrates. In anurans, AVT influences the production of advertisement calls, the acoustic signals that males use to attract females and repel rival males. In this study, we investigate the effects of AVT on call characteristics in the túngara frog (*Physalaemus pustulosus*). Túngara frogs produce a "whine" that is important for species recognition; they may also produce a second, attractive call component, the "chuck". We used a field playback experiment to determine changes in male calling behavior following treatment with AVT. A previous study showed that AVT alters call rate and the production of chucks; in the current analysis, we focus on changes in the whine. Males produce shorter whines with higher initial frequencies following treatment with AVT. Call changes do not vary with a social stimulus. We also used female phonotaxis experiments to investigate the effects of call changes on female mate choice. Females disfavor the calls produced by males treated with exogenous AVT. We suggest that AVT influences motivation to call and the motor control of call production, but that overstimulation of the vocal system limited the production of attractive calls in this experimental context.

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1. Introduction

The peptide hormone arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin modulate a variety of social behaviors in vertebrates (reviewed in Goodson and Bass, 2001; Marler et al., 2003; Yamaguchi and Kelley, 2003). In anurans, males produce acoustic communication signals that mediate male–male interactions and female mate choice (reviewed in Wells and Schwartz, 2007). Several studies of male vocal behavior, together with localization studies of AVT and its receptors in the anuran brain, suggest that AVT influences sensorimotor and motivational systems involved in signal production (reviewed in Rose and Moore, 2002; Wilczynski et al., 2005).

In frogs, treatment with exogenous AVT stimulates the production of advertisement calls, the signals produced by males to make their presence known to other males and females. Males of several species are more likely to produce calls, or produce calls at a greater

rate following treatment with AVT (Boyd, 1994a; Propper and Dixon, 1997; Chu et al., 1998; Klomberg and Marler, 2000; Burmeister et al., 2001; Ten Eyck, 2005; Kime et al., 2007). Treatment with AVT also influences the acoustic characteristics of calls. In cricket frogs (*Acris crepitans*) and green tree frogs (*Hyla cinerea*), for example, males produce calls with higher dominant frequencies after treatment with AVT (Chu et al., 1998; Burmeister et al., 2001). In the gray tree frog (*Hyla versicolor*), males increase call length and the number of pulses per call (Klomberg and Marler, 2000; Trainor et al., 2003). Male túngara frogs (*Physalaemus pustulosus*) add additional components to their calls (Kime et al., 2007, see below). Such changes in calls and calling behavior suggest that AVT influences the motivation of males to call.

In other taxa, the effects of AVT on behavior can depend on the presence of social stimuli (reviewed in Goodson and Bass, 2001; Semsar et al., 2001; Bester-Meredith et al., 2005; Thompson et al., 2008); this has been less studied in anurans. Trainor et al. (2003) showed that in gray tree frogs AVT alters specific call characters only when other males are calling nearby, suggesting that AVT modulates a male's response to sensory information from his social environment. In cricket frogs, AVT inhibits males from lowering their dominant frequency in response to a simulated intruder and from reducing call rate in the time period following

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the termination of a playback stimulus (Chu et al., 1998). A similar effect on call rate is seen in túngara frogs (Kime et al., 2007).

Despite these numerous studies documenting how AVT influences the acoustic characteristics of anuran advertisement calls, very little is known about the salience of AVT-induced call changes to receivers. Indirect evidence suggests that these changes might be meaningful. In the Puerto Rican coqui (*Eleutherodactylus coqui*), for example, females were observed investigating AVT-treated males (Ten Eyck, 2005). Female gray tree frogs have also been observed approaching AVT-injected males (Marler, unpublished data). Nevertheless, the attractiveness of such calls to females has not been investigated directly.

The male túngara frog produces an advertisement call that varies with the presence or absence of other males in the area (Rand and Ryan, 1981; Ryan, 1985). Male túngara frogs produce an amplitude-modulated frequency sweep that we call a 'whine,' which is necessary for mate recognition (Fig. 1). In response to conspecific calls, males add a variable number of 'chucks' to their calls; chucks increase a male's attractiveness to females (Ryan, 1980; Rand and Ryan, 1981). Numerous previous studies show that the temporal and spectral characteristics of the whine are important to females for both species recognition and mate choice (Rand et al., 1992; Wilczynski et al., 1995; Bosch et al., 2000; Ryan and Rand, 2003). In general, alterations in the acoustic characteristics of the whine are much more likely to disrupt female phonotaxis than are changes in the chuck.

In field experiments, treatment with exogenous AVT promotes calling behavior in male túngara frogs, increasing the probability that individuals will call after handling. Arginine vasotocin also increases the production of chucks and decreases the suppression of calling after termination of a playback stimulus (Kime et al., 2007). These results suggest that AVT influences the motivation of males to call. Because previous studies demonstrate that female túngara frogs prefer calls with chucks and higher call rates (Rand and Ryan, 1981; Bosch et al., 2000), these results also suggest that males may

produce more attractive calls after treatment with AVT. Other aspects of the call change as well, however, and it cannot be assumed that an increased number of chucks in AVT-treated males will necessarily result in more attractive calls. This hypotheses needs to be tested directly, as we do here.

In this study, we expand upon previous work to investigate the effects of AVT on the acoustic characteristics of the túngara frog whine, and the effects of these AVT-induced call changes on female choice. In a field experiment, we recorded male calling behavior before and after treatment with exogenous AVT. We used playback stimuli to test whether whine changes following AVT treatment are contingent on the presence of social stimuli. We then used the calls produced by our experimental males in a laboratory phonotaxis study to empirically test whether AVT-induced whine changes make calls more or less attractive to females.

2. Methods

We used a within-subjects experimental design to assess changes in the acoustic characteristics of the túngara frog whine following treatment with AVT, as well as the role of social stimulation on these call changes. Males were captured, placed in a small enclosure in the field, recorded in a playback experiment, injected with AVT, and recorded again. We also conducted between-subjects analyses of call differences among males injected with three different doses of the hormone and a saline control. Finally, we used female phonotaxis experiments to examine the effects of call changes on female mate choice.

2.1. Playback and hormone treatment experiments

We conducted playback experiments with males during June and July 2003, using túngara frog males from populations near Gamboa, Panama. We captured male túngara frogs between 19:00 and 24:00 h, and placed them in small (17 cm diameter) cylindrical enclosures constructed of plastic mesh "gutter guard". These were open to air and water on all sides and were acoustically transparent to sounds in the frequency range of the túngara frog call (see Burmeister et al., 2001 for another study using similar enclosures). Enclosures were placed in shallow water near the perimeter of the small pools from which túngara frogs normally call, at a distance of 2–5 m from other calling males. In using enclosures, we could restrict the focal male's immediate social environment by preventing his movement within the chorus. In addition, males were handled both before and after treatment with AVT, thus reducing a potential confound between the effects of handling stress and the effects of AVT on male calling behavior. Males who failed to call within 15 min of initial capture and enclosure were not tested and were returned to the chorus.

We recorded males using a Marantz (PMD 430) tape recorder and Sennheiser (ME 67) microphone. Playback stimuli were broadcast from a Marantz recorder and a Mineroff (SME-AFS) or Radio Shack (32-2040) speaker placed approximately 0.5 m from the calling male. The playback stimulus was a synthetic conspecific whine, created using software written by J. Schwartz with stimulus parameters from Ryan and Rand (1999). The stimulus was presented at a rate of one call per two seconds, broadcast at a peak amplitude of 80 dB SPL (re. 20 μPa) measured at the calling site of the focal male (Radio Shack 33-2255 SPL meter).

We began pre-injection playback experiments after males resumed calling within the enclosures. We followed an A-B-A experimental design (McGregor et al., 1992). We recorded males for 3 min prior to beginning a playback stimulus (baseline recording), for 3 min during playback of the synthetic conspecific whine (playback recording), and for 3 min after the stimulus was terminated (post-playback recording).

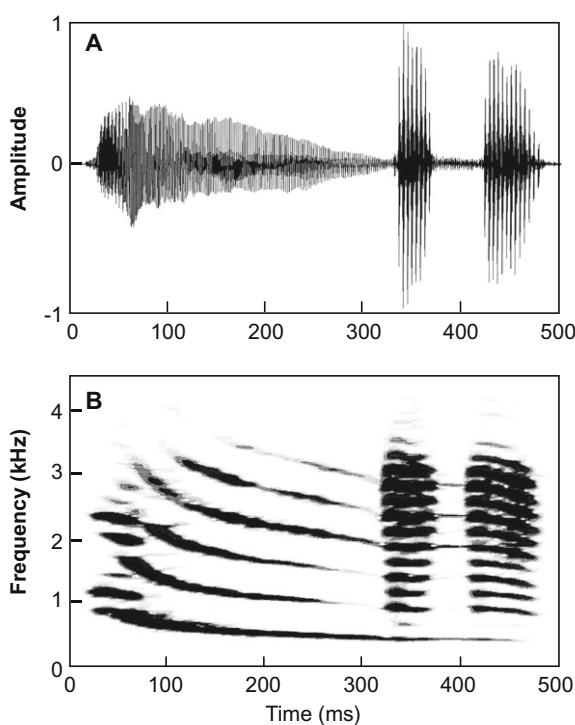


Fig. 1. A typical túngara frog call, with a whine plus two chucks. (A) Oscillogram showing changes in amplitude over time. (B) Spectrogram showing the frequency sweep of the whine and the harmonic structure of the whine and chuck.

Immediately following the pre-injection recording, we removed males from enclosures and injected them subcutaneously with either AVT or a saline control. Control animals ($n = 17$ males) were injected with 25 μ l of saline. Males in the primary treatment group ($n = 18$ males) were injected with 25 μ g of AVT in 25 μ l of saline (the AVT-25 treatment group). Similar doses of AVT have been used in other studies, including studies with cricket frogs, which are similar in size to túngara frogs (Marler et al., 1995; Chu et al., 1998). To assess the effects of AVT dose on calling behavior, we also included two additional doses of AVT in our experimental design: 12 μ g of AVT in 25 μ l of saline ($n = 9$ males) and 50 μ g in 25 μ l of saline ($n = 10$ males). Each night, we randomly assigned each focal male to one of these four treatment groups by drawing from a coded group of syringes.

We immediately returned males to their enclosure, and noted the elapsed time between injection and first call. We began post-injection recordings 30 min after treatment; these followed the same A-B-A (baseline, playback, post-playback) design as the pre-injection recordings. There were therefore a total of six recording periods for each individual: pre-injection baseline, pre-injection playback, pre-injection post-playback, post-injection baseline, post-injection playback, and post-injection post-playback. After the post-injection recording, males were released at their original calling site. We recorded the date of the experiment, the approximate distance to other males in the chorus, chorus size, temperature, and male size (snout-vent length, SVL).

2.2. Call analysis

The temporal structure of a typical túngara frog whine consists of a rapid rise in amplitude followed by a longer, exponential amplitude fall (Fig. 1A, Ryan, 1985). The dominant frequency of the whine sweeps exponentially from high to low frequency; whines also have a number of harmonics (Fig. 1B). Occasionally, whines contain a short “prefix” that precedes the exponential amplitude and frequency drop (Fig. 1B). The temporal and spectral structure of these prefixes vary substantially among males. Whines may also be followed by one or more “chucks”, which males produce in social interactions with other males (Rand and Ryan, 1981).

We digitized field recordings using Cool Edit 96 at a sampling rate of 22050 Hz. To analyze calls, we used custom batch processing algorithms created in Matlab for the purpose of this study. We report data on eight call variables that are typically used to describe the temporal and spectral characteristics of the túngara frog whine (e.g. Ryan and Rand, 2001). Chuck production and rates of calling were not analyzed for this paper, but are reported elsewhere (Kime et al., 2007).

Temporal call characters were measured from the amplitude envelope of the whine. We measured **whine duration** as the time from 10% of maximum amplitude on the rising portion of the whine to 10% of maximum amplitude on the fall. Whine duration included prefixes when present but not chucks, which are usually considered to be a separate call component. Chucks sometimes overlap the end of the whine; in these cases we defined the end of the whine as the point of minimum amplitude preceding the chuck. Because whine duration was not correlated with chuck production in our data set ($r = -0.250$, $p = 0.388$, $n = 14$), we assume that differences in whine duration did not reflect differences in chuck production. **Rise time** was measured from 10% to 90% of maximum amplitude at the beginning of the whine. Rise times included prefixes, when present. **Time at maximum amplitude** was measured between 90% of maximum amplitude on the rise and 90% of maximum amplitude on the fall. **Fall time** was measured from 90% to 10% of maximum amplitude on the fall portion of the whine. **Fall shape** was calculated as the proportion of the fall time that it takes to reach 50% of maximum amplitude; exponen-

tial amplitude falls in túngara frogs typically have fall shapes <0.5 . We did not measure rise shape, as this was highly variable among individuals, in part because of variability in the presence or absence of the prefix.

Spectral measurements were taken from a frequency contour drawn through the fundamental of the túngara frog whine. We did not measure the harmonics of the whine, as previous phonotaxis experiments demonstrate that the upper harmonics of the whine do not influence female choice (Rand et al., 1992; Wilczynski et al., 1995). For this study, we defined the **initial frequency** of the whine as the frequency at 90% amplitude on the whine's rise. Initial frequency never included the prefix, and was significantly correlated with the frequency at maximum amplitude and with dominant frequency (unpublished data). **End frequency** was defined as the frequency at 10% of the maximum amplitude on the fall of the whine. We also measured **frequency shape** as the proportion of time to reach the midpoint between the initial and end frequency. As with fall shape, exponential frequency sweeps in túngara frogs typically have frequency shape values <0.5 .

2.3. Statistical analysis of male behavior

For the AVT-25 males (our primary treatment group), we used a two-way repeated measures analysis of variance to assess differences in each of the measured whine characteristics (1) among the three playback periods and (2) between pre- and post-injection recordings. Testing for an effect of playback period allowed us to determine whether males alter their whines in the presence of a social stimulus; comparing pre- and post-injection recordings tested for the effect of AVT on whine characteristics. To determine whether the effects of AVT varied with social context, we tested for interactions between the effects of AVT treatment and playback period on whine characteristics. Only those AVT-25 males that called in all six pre- and post-injection recording periods were entered into this within-subjects analysis ($n = 10$ of the 18 recorded AVT-25 males).

For our dose-response analysis, we were primarily interested in determining whether different doses of AVT had different effects on male calling behavior. We therefore restricted this analysis to the baseline (no playback) recording period. Because many males did not call in all recording periods, especially in the saline treatment group, restricting analysis to baseline recordings also increased sample size. We performed a one-way repeated measures analysis of variance to assess the effects of AVT on each of the measured whine characteristics in the saline, AVT-12, AVT-25, and AVT-50 treatment groups. This was a within-subjects analysis. In a between-subjects analysis, we used an analysis of covariance to compare post-injection call characteristics among the saline, 12 μ g, 25 μ g, and 50 μ g treatment groups. Pre-injection whine characteristics were used as a covariate, and we used Bonferroni-corrected pairwise post hoc tests to compare doses when the overall analysis demonstrated significant differences among the four treatment groups. Sample sizes for the dose response analyses were as follows: $n = 4$ saline males, $n = 7$ AVT-12 males, $n = 14$ AVT-25 males, $n = 9$ AVT-50 males. Since more AVT-25 males called during these two baseline periods than during all six periods of the playback study, the sample size for AVT-25 males differed from the full analysis described above.

In the AVT-25 treatment group, there were few consistent correlations between whine variables and date, time, or temperature (unpublished data). In the pre-injection recordings, beginning, but not end, frequency was correlated with male size (snout-vent length, SVL) ($r = -0.572$, $p = 0.041$, baseline recording period). Given the within-subjects design of this study, and because the between-subjects analysis used pre-injection call characters as a covariate, we used uncorrected data in all statistical analyses.

2.4. Female phonotaxis experiments

Two dichotomous-choice phonotaxis experiments were used to assess the effects of AVT-induced whine changes on female mating preferences. In one experiment, females were tested for their preferences with respect to typical pre- and post-injection whines from AVT-25 males. For each of the 14 males that called both before and after treatment, we selected one pre- and one post-injection call exemplar from the center of a male's calling bout. Chucks were trimmed from the calls to test specifically for the effects of whine changes on the attractiveness of calls to females. Each female ($n = 14$) was presented with one paired set of stimuli from a particular male. We used a two-tailed binomial test to determine whether the group of females preferred one stimulus over another.

In a second experiment, females were tested for preferences with respect to complete calls produced before and after injection with AVT, including chucks when present. The call exemplars used in this experiment were the same as those noted above, except that chucks were not trimmed from the call. In the fourteen pairs of males stimuli, seven of the pre-injection calls and ten of the post-injection calls had at least one chuck. As above, each female ($n = 14$) was tested with one paired set of stimuli from a particular male; these were not necessarily the same females as those tested with the whine-only stimuli. We used a two-tailed binomial test to determine whether the group of females showed a preference for pre- or post- injection calls.

Female choice experiments were conducted between June and August 2004. Females were collected in the field in amplexus and transported to our acoustics lab at facilities of the Smithsonian Tropical Research Institute. We conducted the phonotaxis experiments in an Acoustic Systems (Austin, TX) sound attenuation chamber that measured 1.8 m by 2.7 m. The female's behavior was observed on a video monitor equipped with an infrared light source connected to a wide-lens video camera. The camera was located on the chamber's ceiling. We placed a female in the center of the chamber under a small cone that could be raised remotely to initiate testing. We broadcast the test stimuli from ADS speakers, one each in the center of the wall opposite one another, such that the peak amplitude of the whine of each test call at the center of the arena was 82 dB SPL (re. 20 μ Pa). The two calls presented to each female were broadcast antiphonally at a rate of one call per two seconds from each speaker, a typical calling rate for male túngara frogs. A positive phonotactic response was noted if a female approached within 10 cm of one of the speakers as long as this response did not result from the female following the chamber's walls. A "no response" was recorded if a female remained motionless for the first five min or any subsequent two min of the trial, or if she did not exhibit phonotaxis after 15 min. Females were returned to their site of capture that same night.

2.5. Ethical statement

To avoid re-testing males and females captured on multiple nights, we marked individuals with a unique toeclip combination (following the Guidelines for the Use of Live Amphibians and Reptiles in Field Research, compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR), available at <http://www.asih.org>). Toeclipping does not appear to influence survival in this species, as toe-clipped individuals were recaptured in the field over the course of the experiment. Experimental protocols were approved by the University of Texas Institutional Animal Care and Use Committee, Panama's Autoridad Nacional del Ambiente (ANAM), and the UW Madison Institutional Animal Care and Use Committee.

3. Results

3.1. Within-male changes in AVT-25 males

Ten AVT-25 males called in all six recording periods. **Table 1** shows the mean (\pm SD) value for each call variable measured from these 10 males. We used a two-way repeated measures analysis of variance to assess within-male changes in whine characteristics (1) among playback periods and (2) after injection with AVT. There was no effect of playback period on any of the eight measured whine variables ($P > 0.1$ for all variables). There was a significant effect of AVT on five of the eight measured whine variables (call duration, $P < 0.005$; time at max, $P < 0.05$; fall time, $P < 0.001$, initial frequency, $P < 0.01$, and frequency shape, $P < 0.005$). After injection with AVT, túngara frog males generally produced shorter whines with shorter fall times but longer times at maximum amplitude (Fig. 2A). Post-injection whines had higher initial frequencies, although end frequency did not change with AVT treatment (Fig. 2B). The frequency sweep of the whine was more linear, and less exponential, after treatment (Fig. 2C). Fig. 3 shows a typical call from one male pre- and post-injection. There were no significant interactions between the effects of playback stimulus and AVT ($P > 0.05$ for all variables).

3.2. Dose-response experiments

For the dose-response analysis, we used only the baseline recordings of males prior to and following treatment with AVT or a saline control. Sample sizes were as follows: $n = 4$ saline, $n = 7$ AVT-12, $n = 14$ AVT-25, and $n = 9$ AVT-50 males.

3.2.1. Within-subjects analysis of call changes

We used a one-way repeated measures analysis of variance to assess within-subjects call changes after treatment with saline or one of four doses of AVT. Significant changes in the whine between pre- and post-injection recordings were limited to AVT males (Fig. 4). There were no significant treatment effects for any whine variable in the saline males, although a small sample size increased the likelihood of Type II error. In the AVT males, there were consistent changes in all three treatment groups with respect to call

Table 1

Whine measurements for túngara males before and after treatment with AVT. For each variable, top row, pre-injection recordings; bottom row, post-injection recordings. BL, baseline; PB, playback; and PPB, post-playback recording periods. $N = 10$ males.

	Whine measurements (mean \pm SD)		
	BL	PB	PPB
Duration (ms)	299 \pm 58	299 \pm 44	309 \pm 40
Duration (ms)	252 \pm 48	261 \pm 49	277 \pm 44
Rise time (ms)	22 \pm 20	23 \pm 21	22 \pm 18
Rise time (ms)	39 \pm 32	36 \pm 29	34 \pm 22
At max (ms)	18 \pm 7	18 \pm 9	19 \pm 7
At max (ms)	25 \pm 11	24 \pm 9	25 \pm 9
Fall time (ms)	259 \pm 52	257 \pm 34	268 \pm 33
Fall time (ms)	188 \pm 39	201 \pm 42	218 \pm 50
Fall shape	0.35 \pm 0.18	0.35 \pm 0.14	0.38 \pm 0.16
Fall shape	0.42 \pm 0.15	0.41 \pm 0.13	0.42 \pm 0.15
Initial freq (Hz)	820 \pm 48	820 \pm 54	813 \pm 54
Initial freq (Hz)	880 \pm 69	873 \pm 68	874 \pm 58
End freq (Hz)	489 \pm 39	482 \pm 36	480 \pm 31
End freq (Hz)	505 \pm 42	494 \pm 48	480 \pm 34
Freq shape	0.28 \pm 0.05	0.28 \pm 0.06	0.27 \pm 0.05
Freq shape	0.41 \pm 0.07	0.40 \pm 0.08	0.40 \pm 0.09

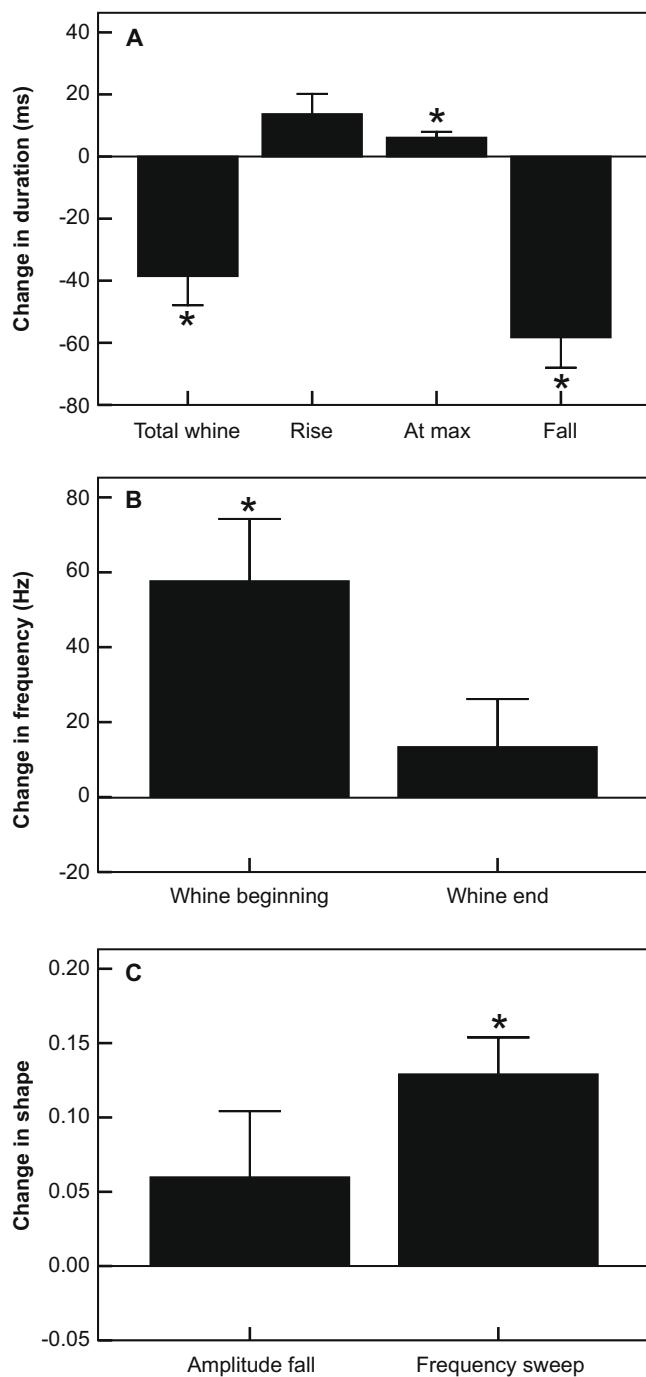


Fig. 2. Changes in whine characteristics following treatment with 25 µg AVT. (A) whine duration, (B) fundamental frequency, and (C) the shape of the whine's amplitude fall and frequency sweep. Bars represent the mean ($\pm 1\text{SE}$) within-subject difference between pre-injection and post-injection calls produced during the baseline recording period. Asterisks show significant effects of AVT (repeated measures ANOVA, $P < 0.05$). $N = 10$ males.

duration, amplitude fall time, initial frequency, and frequency shape (Fig. 4, $P < 0.05$ for each of these call variables in AVT-12, AVT-25, and AVT-50 males). After treatment with AVT, males decreased whine duration and had shorter amplitude fall times; they also increased the initial frequency of the call, and had more linear decreases in frequency. Significant changes in other call variables occurred in some treatment groups but not others (Fig. 4). In several of the AVT-50 males, longer rise times reflected the production of unusually long call prefixes.

3.2.2. Between-subjects analysis of call differences among treatment groups

We used an analysis of covariance to compare post-injection call characteristics among the four treatment groups. There were significant differences among the treatment groups in post-injection calls for two of eight whine variables: beginning frequency ($P < 0.05$) and frequency shape ($P < 0.005$). Bonferroni-corrected post hoc analysis showed that differences occurred only between the saline and AVT males. Saline males had a lower initial frequency than AVT-12 males ($P < 0.05$). There were significant differences in frequency shape between saline males and all AVT treatment groups (saline versus AVT-12: $P < 0.001$, saline versus AVT-25: $P < 0.010$, saline versus AVT-50: $P < 0.005$). There were no significant pairwise differences in post-injection whine characteristics between any of the three AVT treatment groups.

3.3. Female phonotaxis experiments

In phonotaxis experiments, females preferred whines produced prior to injection with AVT to whines of the same males produced after injection. When given a choice between pre- and post-injection recordings of AVT-25 whines, 12 of 14 females chose the pre-injection call (two-tailed binomial $P = 0.0129$). When chucks were included, 10 of 14 females chose the pre-injection call ($P = 0.180$).

4. Discussion

Male túngara frogs change a suite of spectral and temporal advertisement call characteristics after treatment with exogenous arginine vasotocin (AVT). This includes an increase in the initial frequency and a decrease in the duration of the “whine” component of their call. The production of calls with such characteristics decreases the whine’s attractiveness to females. We consider observed changes in whine characteristics to what is currently known about vocal production and mate choice in this species, as well as recent hypotheses about the general effects of AVT on anuran calling behavior. We suggest that exogenous treatment with AVT likely influences túngara frog call production via several pathways, including motivation to call and the modulation of air movement through the vocal tract. We also suggest that the decreased attractiveness of AVT-induced calls may result from over-stimulation of the vocal system, reflecting limitations on the physical structures that support the production of attractive calls.

A number of behavioral studies have demonstrated that treating male frogs with AVT influences both the probability of calling and specific characteristics of advertisement calls (reviewed in Moore et al., 2005; Wilczynski et al., 2005). Correspondingly, AVT receptors are found in several brain areas related to the control of vocal behavior (reviewed in Emerson and Boyd, 1999; Wilczynski et al., 2005). These combined data suggest at least three possible mechanisms by which calling could be modulated by the action of AVT in the central nervous system. One is to increase the motivation to call. The presence of AVT and AVT receptors in forebrain areas such as the striatum, amygdala, and pre-optic area supports this hypothesis for anurans, as do behavioral data that show an increase in the probability of calling after AVT injections (e.g. Chu et al., 1998; Burmeister et al., 2001). The fact that AVT increases the probability of calling after injection suggests that this is true for túngara frogs as well (Kime et al., 2007).

A second possible target for AVT is brain areas upstream of the motor output regions that control contraction of the oblique muscles, such as the pretrigeminal nucleus (Boyd, 1994b; Emerson and Boyd, 1999; reviewed in Wilczynski et al., 2005). Whether AVT can alter the pattern of muscle contraction has, to our knowledge, not been explicitly tested in frogs. In midshipman fish, however, the rhythmic motor activity of vocalizations is influenced by AVT,

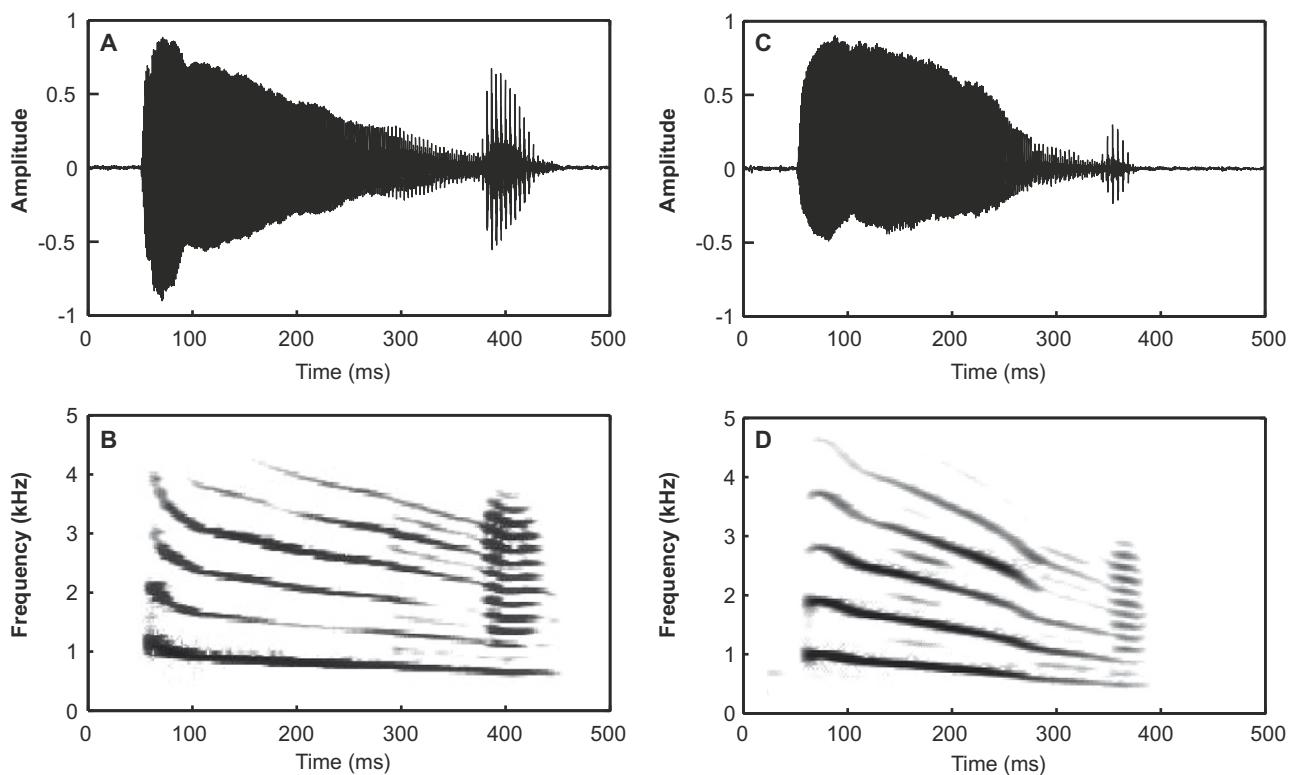


Fig. 3. (A) Oscillogram and (B) sonogram of a pre-injection whine + chuck. (C) Oscillogram and (D) sonogram of a call from the same male after injection with AVT.

and the pretrigeminal nucleus is a central component of the vocal central pattern generator in amphibians (Goodson and Bass, 2000; Bass and Remage-Healey, 2008).

In túngara frogs, airflow influences both call duration and frequency. The production of calls in túngara frogs occurs when males use abdominal muscles to contract the lungs and thus push air past the vocal folds in the larynx. Rapid movement of air past the vocal folds at the beginning of the call produces the high amplitude, high frequency portion of the whine (Martin, 1972; Dudley and Rand, 1991; Pauly et al., 2006; Suthers et al., 2006). As air flow slows near the end of the whine, both the amplitude and the frequency of the call decrease. The whine appears to be terminated with slowing airflow (Gridi-Papp, personal communication). In a recent study, Pauly et al. (2006) showed that males produce calls that are shorter in duration and have a lower initial frequency during initial call bouts, when lung inflation is not yet at its maximum. In the current study, an AVT-mediated increase in initial air pressure past the vocal folds could have caused the observed call changes of higher initial frequency and shorter duration without altering the frequency at the end of the call.

Additional evidence that AVT influences airflow comes from observations of the chucks produced by treated males. In some cases, AVT-treated males have chucks that are of low amplitude (unpublished data, see Fig. 3). Chucks are instigated by a concurrent increase in air pressure and movement of a cartilaginous mass into the stream of air flow (Gridi-Papp et al., 2006). Low amplitude chucks could be another indication that males are pushing an excess of air through the larynx at the beginning of the whine, resulting in a depletion of air available to produce the chucks that follow it. This, also, could influence female mate choice, as females show preferences for chucks only when the amplitude of the chuck is higher than the amplitude of the whine (Rand, unpublished data). Thus, while AVT increases the motivation to produce chucks (Kime et al., 2007), it can also decrease the ability of males to use this call component in attracting females.

A third possible effect of AVT on call production may occur via the modulation of responses to social stimuli (e.g. Rose and Moore, 2002). In several amphibian species, AVT appears to play a role in sensory processing of social stimuli (Goodson and Bass, 2001; Rose and Moore, 2002). In roughskin newts, for example, AVT enhances responses toward visual cues (Thompson and Moore, 2000) and amplifies somatosensory information that induces clasping (Rose and Moore, 2002; Thompson et al., 2008). In gray tree frogs, AVT-induced call changes occur only in the context of social stimulation, suggesting that AVT increases selective attention to conspecific calls (Trainor et al., 2003). This appears not to be the case for túngara frogs, at least with respect to the social stimulus of conspecific calls. In a previous study, males treated with AVT increased chuck production both in the presence and absence a conspecific playback stimulus (Kime et al., 2007). In this study, we likewise demonstrate that changes in the whine do not vary with the presentation of such a social stimulus. Whether AVT influences the response of males to other stimuli, such as the presence of females, has yet to be explored.

We used dose-response experiments to determine whether changes in calling behavior were specific to a particular treatment level of AVT. The general effects of AVT on the characteristics of the túngara frog whine did not depend on the dose given; there appears to be a threshold effect below 12 µg of AVT (see also Kime et al., 2007). One of the problems associated with exogenous treatment, however, is that we do not know how much AVT is getting to areas in the central nervous system, where exactly it is acting, and whether peripheral receptors for AVT might influence vocal behavior. Central injections of AVT in this model system would clarify some of these issues.

In the current study, females showed a preference for pre-injection whines over post-injection whines from the same male. This result, although initially surprising, is supported by previous phototaxis data on call preferences in female túngara frogs. Female túngara frogs use final frequency in species recognition, and rise

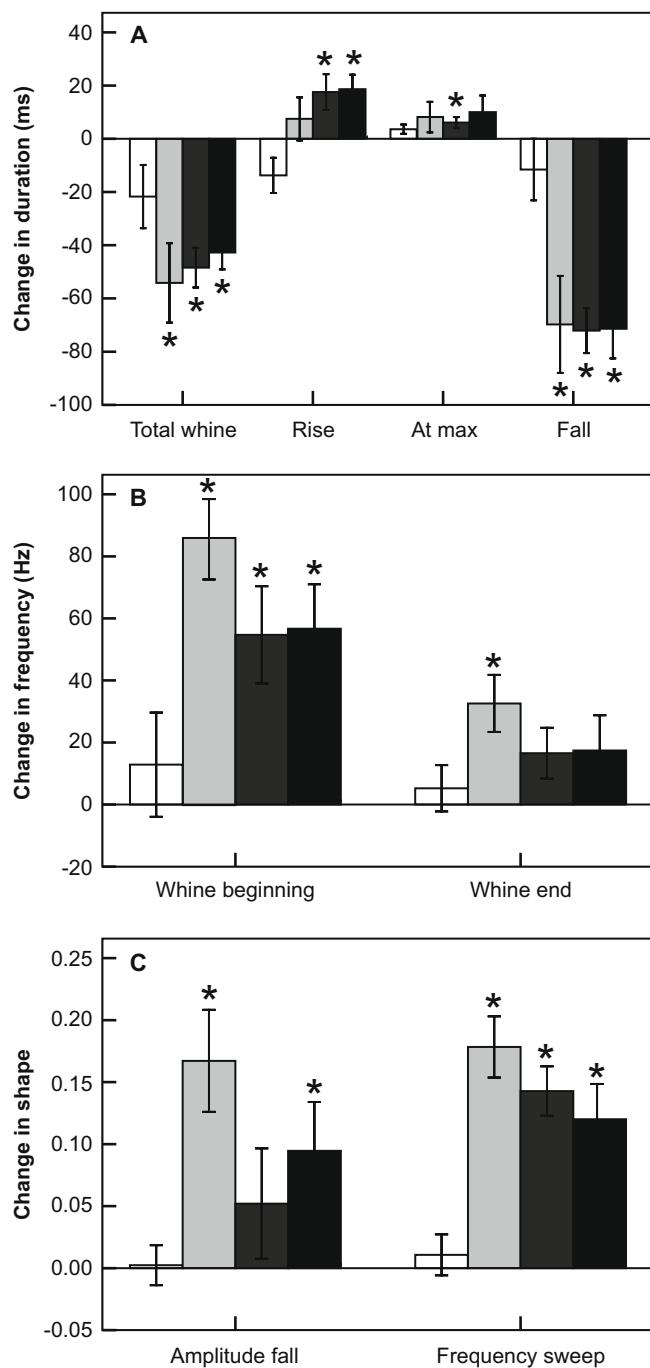


Fig. 4. Within-male changes in whine characteristics following treatment with AVT or a saline control in dose-response experiments. (A) whine duration, (B) fundamental frequency, and (C) the shape of the whine's amplitude fall and frequency sweep. Bars represent the mean (\pm SE) within-subject difference between pre-injection and post-injection calls produced during the baseline recording period (for left to right) saline males ($n = 4$), AVT-12 males ($n = 7$), AVT-25 males ($n = 14$), and AVT-50 males ($n = 9$). Asterisks show significant effects of treatment (repeated measures ANOVA, $P < 0.05$).

shape, fall shape, and frequency shape in discrimination (Ryan and Rand, 1999). In addition, female túngara frogs show a weak preference for calls with lower dominant frequency (Ryan, 1980; Wilczynski et al., 1995; Bosch et al., 2000). Exogenous treatment with AVT may therefore have caused males to produce calls with numerous characteristics that are less attractive to females. For example, one possibility is that the calls of AVT-treated males had frequency shapes outside the range of normal conspecific var-

iation; in our study, the linear frequency sweep of AVT-induced calls was more than 2 SD from the pre-injection mean for this variable (see Fig. 3 and Table 1). The higher initial frequency of AVT calls may have also influenced female choice.

In a previous study, we suggested that túngara frog males with higher levels of AVT may be more attractive to females because they produce more chucks (Kime et al., 2007). Interestingly, this was not the case in the phonotaxis experiments that we report here. Increased chuck production may not be enough to compensate for the decreased attractiveness of the whine, especially if chucks are of low amplitude relative to the whine (as noted above). In summary, our results support the idea that AVT increases sexual motivation in males, but that being motivated does not necessarily correspond with being more attractive. Such a fitness cost could be compensated for if males with higher levels of AVT are more effective in male-male acoustic interactions, but we do not know at present how AVT might function in that arena.

In conclusion, treating male túngara frogs with AVT influences motivation to call and changes call characteristics in a way that likely reflects increased air flow through the vocal system. These behavioral data are consistent with known distributions of AVT receptors in the anuran brain. Such hormone manipulations cause males to produce advertisement signals that are not attractive to female receivers, however, reflecting limitations on the physical structures responsible for signal production or tradeoffs in the functions of the túngara advertisement call.

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References

- Bass, A.H., Remage-Healey, L., 2008. Central pattern generators for social vocalization: androgen-dependent neurophysiological mechanisms. *Horm. Behav.* 53, 659–672.
- Bester-Meredith, J.K., Martin, P.A., Marler, C.A., 2005. Manipulations of vasopressin alter aggression differently across testing conditions in monogamous and non-monogamous *Peromyscus* mice. *Aggr. Behav.* 31, 189–199.
- Bosch, J., Rand, A.S., Ryan, M.J., 2000. Signal variation and call preferences for whine frequency in the túngara frog, *Physalaemus pustulosus*. *Behav. Ecol. Sociobiol.* 49, 62–66.
- Boyd, S.K., 1994a. Arginine vasotocin facilitation of advertisement calling and call phonotaxis in bullfrogs. *Horm. Behav.* 28, 232–240.
- Boyd, S.K., 1994b. Gonadal steroid modulation of vasotocin concentrations in the bullfrog brain. *Neuroendocrinology* 60, 150–156.
- Burmeister, S., Somes, C., Wilczynski, W., 2001. Behavioral and hormonal effects of exogenous vasotocin and corticosterone in the green treefrog. *Gen. Comp. Endocrinol.* 122, 189–197.
- Chu, J., Marler, C.A., Wilczynski, W., 1998. The effects of arginine vasotocin on the calling behavior of male cricket frogs in changing social contexts. *Horm. Behav.* 34, 248–261.
- Dudley, R., Rand, A.S., 1991. Sound production and vocal sac inflation in the túngara frog, *Physalaemus pustulosus* (Leptodactylidae). *Copeia* 1991, 460–470.
- Emerson, S.B., Boyd, S.K., 1999. Mating vocalizations of female frogs: control and evolutionary mechanisms. *Brain Behav. Evol.* 53, 187–197.
- Goodson, J.L., Bass, A.H., 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* 403, 769–772.
- Goodson, J.L., Bass, A.H., 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain Res. Rev.* 35, 246–265.
- Gridi-Papp, M., Rand, A.S., Ryan, M.J., 2006. Animal communication: complex call production in the túngara frog. *Nature* 441, 38.
- Kime, N.M., Whitney, T.K., Davis, E.S., Marler, C.A., 2007. Arginine vasotocin promotes calling behavior and call changes in male túngara frogs. *Brain Behav. Evol.* 69, 254–265.
- Klomberg, K.F., Marler, C.A., 2000. The neuropeptide arginine vasotocin alters male call characteristics involved in social interactions in the grey treefrog, *Hyla versicolor*. *Anim. Behav.* 59, 807–812.

- Marler, C.A., Bester-Meredith, J.K., Trainor, B.C., 2003. Paternal behavior and aggression: endocrine mechanisms and nongenomic transmission of behavior. *Adv. Study Behav.* 32, 263–323.
- Marler, C.A., Chu, J., Wilczynski, W., 1995. Arginine vasotocin injection increases probability of calling in cricket frogs, but causes call changes characteristic of less aggressive males. *Horm. Behav.* 29, 554–570.
- Martin, W.F., 1972. Evolution of vocalization in the genus *Bufo*. In: Blair, W.F. (Ed.), *Evolution in the Genus Bufo*, University of Texas Press, Austin, pp. 279–309.
- McGregor, P.K., Catchpole, C.K., Dabelsteen, T., Falls, J.B., Fusani, L., Gerhardt, H.C., Gilbert, F., Horn, A.G., Klump, G.M., Kroodsma, D.E., Lambrechts, M.M., McComb, K.E., Nelson, D.A., Pepperberg, I.M., Ratcliffe, L., Searcy, W.A., Weary, D.M., 1992. Design of playback experiments: the thornbridge hall NATO ARW consensus. In: McGregor, P.K. (Ed.), *Playback and Studies of Animal Communication*. Plenum Press, New York, pp. 1–9.
- Moore, F.L., Boyd, S.K., Kelley, D.B., 2005. Historical perspective: hormonal regulation of behaviors in amphibians. *Horm. Behav.* 48, 373–383.
- Pauly, G.B., Bernal, X.E., Rand, A.S., Ryan, M.J., 2006. The vocal sac increases call rate in the túngara frog *Physalaemus pustulosus*. *Physiol. Biochem. Zool.* 79, 708–719.
- Propper, C.R., Dixon, T.B., 1997. Differential effects of arginine vasotocin and gonadotropin-releasing hormone on sexual behaviors in an anuran amphibian. *Horm. Behav.* 32, 99–104.
- Rand, A.S., Ryan, M.J., 1981. The adaptive significance of a complex vocal repertoire in a neotropical frog. *Z. Tierpsychol.* 57, 209–214.
- Rand, A.S., Ryan, M.J., Wilczynski, W., 1992. Signal redundancy and receiver permissiveness in acoustic mate recognition by the túngara frog, *Physalaemus pustulosus*. *Am. Zool.* 32, 81–90.
- Rose, J.D., Moore, F.L., 2002. Behavioral neuroendocrinology of vasotocin and vasopressin and the sensorimotor processing hypothesis. *Front. Neuroendocrinol.* 23, 317–341.
- Ryan, M.J., 1980. Female mate choice in a neotropical frog. *Science* 209, 523–525.
- Ryan, M.J., 1985. The Túngara Frog: A Study in Sexual Selection and Communication. University of Chicago Press, Chicago.
- Ryan, M.J., Rand, A.S., 1999. Phylogenetic influences on mating call preferences in female túngara frogs (*Physalaemus pustulosus*). *Anim. Behav.* 57, 945–956.
- Ryan, M.J., Rand, A.S., 2001. Feature weighting in signal recognition and discrimination by the túngara frog. In: Ryan, M.J. (Ed.), *Anuran Communication*. Smithsonian Institution Press, Washington, DC, pp. 86–101.
- Ryan, M.J., Rand, A.S., 2003. Sexual selection in female perceptual space. How female túngara frogs perceive and respond to complex population variation in acoustic mating signals. *Evolution* 57, 2608–2618.
- Semsar, K., Kandel, F.L.M., Godwin, J., 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Horm. Behav.* 40, 21–31.
- Suthers, R.A., Narins, P.M., Lin, W.Y., Schnitzler, H.U., Denzinger, A., Xu, C.H., Feng, A.S., 2006. Voices of the dead: complex nonlinear vocal signals from the larynx of an ultrasonic frog. *J. Exp. Biol.* 209, 4984–4993.
- Ten Eyck, G.R., 2005. Arginine vasotocin activates advertisement calling and movement in the Puerto Rican frog, *Eleutherodactylus coqui*. *Horm. Behav.* 47, 223–229.
- Thompson, R.R., Dickinson, P.S., Rose, J.D., Dakin, K.A., Civello, G.M., Segerdahl, A., Bartlett, R., 2008. Pheromones enhance somatosensory processing in newt brains through a vasotocin-dependent mechanism. *Proc. R. Soc. B Biol. Sci.* 275, 1685–1693.
- Thompson, R.R., Moore, F.L., 2000. Vasotocin stimulates appetitive responses to the visual and pheromonal stimuli used by male roughskin newts during courtship. *Horm. Behav.* 38, 75–85.
- Trainor, B.C., Rouse, K.L., Marler, C.A., 2003. Arginine vasotocin interacts with the social environment to regulate advertisement calling in the gray treefrog (*Hyla versicolor*). *Brain Behav. Evol.* 61, 165–171.
- Wells, K.D., Schwartz, J.J., 2007. The behavioral ecology of anuran communication. In: Narins, P.M., Feng, A.S., Fay, R.R., Popper, A.N. (Eds.), *Hearing and Sound Communication in Amphibians*. Springer, New York, pp. 44–86.
- Wilczynski, W., Rand, A.S., Ryan, M.J., 1995. The processing of spectral cues by the call analysis system of the túngara frog, *Physalaemus pustulosus*. *Anim. Behav.* 49, 911–929.
- Wilczynski, W., Lynch, K., O'Bryant, E., 2005. Current research in amphibians: studies integrating endocrinology, behavior, and neurobiology. *Horm. Behav.* 48, 440–450.
- Yamaguchi, A., Kelley, D.B., 2003. Hormonal mechanisms in acoustic communication. In: Simmons, A.M., Popper, A.N., Fay, R.R. (Eds.), *Acoustic Communication*. Springer-Verlag, New York, pp. 275–323.