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Arginine vasotocin affects vocal behavior but not selective responses to conspecific calls in male túngara frogs

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ABSTRACT

Arginine vasotocin (AVT) and its homolog arginine vasopressin (AVP) modulate social behavior, including social communication. In anuran amphibians, male-male competition and female mate choice rely heavily on acoustic signaling. Behavioral experiments show that AVT influences motivation to call and vocal production. It may also influence how males process and respond to socially relevant auditory stimuli, but few studies have explored this possibility in this taxon. Túngara frogs produce a "whine" that is used for species recognition; in competition with other males they append one or more attractive "chucks" to the whine. Frequency modulation in the whine is an important cue for recognizing conspecifics, and gating of conspecific signals begins in the auditory midbrain. We used dynamic playback experiments to investigate the effects of exogenous AVT on males' responses to stimuli with species-typical and altered frequency modulation. We used avoidance of call overlap as evidence that a male recognizes a stimulus as salient and the production of attractive chucks as evidence of his competitive response to a proximate rival. We used call rate, whine duration, and whine frequency as measures of motivation and motor production. Males responded selectively to a stimulus with species-typical frequency modulation. Following treatment with AVT, they increased call rate and altered whines and chucks in a way that suggests increased air flow during the whine. We did not, however, find evidence that treatment with AVT alters the salience of frequency modulation in recognizing and responding to acoustic signals, at least for the stimuli used in this study.

1. Introduction

The neuropeptide hormone arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin (AVP) influence social interactions in vertebrate animals by modulating an individual's responses to external and internal cues (Goodson and Bass, 2001). The impacts of AVT/AVP on social behavior vary among species and contexts, and it appears to influence multiple interacting neural pathways. Studies across vertebrate taxa demonstrate that AVT/AVP fine-tunes sensory processing, modulates the social decision-making network, and alters motor output (reviewed in Albers, 2012; Bester-Meredith et al., 2015; Rose and Moore, 2002; Wilczynski et al., 2017).

In anuran amphibians (frogs and toads), social interactions are mediated by acoustic communication. Males produce advertisement and aggressive calls to attract females and interact with other males. They modify vocal production in response to incoming sensory information, including the calls of conspecific competitors. As in other vertebrates, AVT may modulate male-male interactions by altering sensory processing, motivation to call, and the motor control of vocal production (Wilczynski et al., 2017, 2005). There is substantial evidence that AVT influences motivation to call and the motor control of vocal production in male anurans (Boyd, 2013; Wilczynski et al., 2017). For example, treatment with exogenous AVT increases the likelihood that males will call in cricket frogs (*Acris crepitans*), coqui frogs (*Eleutherodactylus coqui*), and western clawed frogs (*Xenopus tropicalis*) (Marler et al., 1995; Miranda et al., 2015; Ten Eyck, 2005). In bullfrogs (*Rana catesbeiana*), injections of vasotocin into the laryngeal motor nuclei induce calling (Boyd, 2013).

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But fewer experiments in this taxon have investigated how AVT might alter the processing of and behavioral responses to auditory stimuli from conspecific rivals. AVT cells and fibers are widespread in the anuran brain, including in auditory processing areas and the social decision-making network. This includes the torus semicircularis (Boyd, 2013, 1997), where experimental treatment with AVT alters sensitivity to acoustic stimuli in some green treefrogs (Penna et al., 1992). The behavioral effects of AVT depend on the acoustic context provided by other males. In gray treefrogs, for example, males treated with AVT produce longer calls only when a calling conspecific is nearby, suggesting that it enhances attention to conspecific signals (Trainor et al., 2003). To our knowledge, however, no studies have investigated how AVT changes the selectivity with which male amphibians respond to the signals of conspecific competitors. If AVT changes the processing of sensory stimuli, it may alter the frog's perception of which signals are socially relevant (sensu Ryan and Rand, 1993).

In the túngara frog, *Physalaemus* (=*Engystomops*) *pustulosus*, males produce advertisement calls that vary with immediate social circumstance (Rand and Ryan, 1981; Ryan, 1985). They produce an amplitude and frequency modulated advertisement call, the "whine", which is critical for species recognition. In response to calls of other males, they may add a variable number of "chucks" to their whine (Fig. 1). Female túngara frogs prefer whines with added chucks (Rand and Ryan, 1981; Ryan, 1980) and more chucks to fewer chucks (Akre et al., 2011), but chucks also increase the risk of predation and parasitism (Bernal et al., 2006; Ryan et al., 1982).

In male-male interactions, túngara frogs respond dynamically to conspecific calls. Frequency modulation is an important cue for conspecific recognition. Stimuli with species-typical high to low frequency modulation elicit a greater response in terms of both whines and chucks than stimuli in which frequency modulation is reversed (Ryan, 1983; Zelick et al., 1991), although males are more permissive in their responses to signal variation than females (Bernal et al., 2009, 2007). Males avoid overlapping their calls with conspecific neighbors (Greenfield and Rand, 2001), perhaps because females prefer leading calls (Ryan, unpublished data). Males also respond with more chucks to conspecific stimuli that do not overlap their own, perhaps in part because they can "hear" the characteristics of their rivals' calls (Schwartz and Rand, 1991). In dynamic playback experiments, males de-escalate chuck production when a computer "rival" de-escalates by producing fewer chucks (Goutte et al., 2010).

Arginine vasotocin influences the behavior of male túngara frogs. When treated with AVT in the field, males are more likely to call following injection and produce calls with more chucks during spontaneous calling, suggesting that AVT increases motivation to call and changes a male's response to the cost-benefit tradeoffs of attractive signal production (Kime et al., 2007). Males who have been treated with AVT also produce whines that are shorter and have higher frequencies (Kime et al., 2010). These changes in the whine might be due to increased air flow during calling, reflecting changes in motivation to call and/or the motor control of vocal production (Kime et al., 2019, 2010). Males treated with AVT also respond more quickly with phonotaxis to a recorded chorus than males treated with a saline control (Baugh and Ryan, 2017).

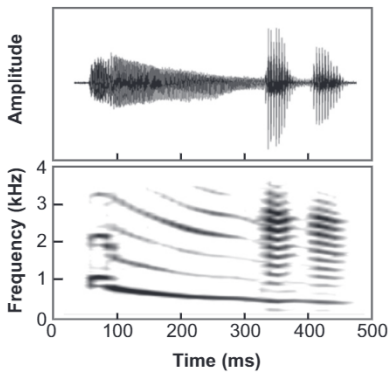

Fig. 1. Oscillogram (top) and spectrogram (bottom) of a túngara frog whine-chuck.

Although there is some understanding of how AVT influences motivation to call and vocal production in male túngara frogs, little is known about how this neuropeptide influences the processing of and behavioral response to a rival's signals. In this study, we investigated whether AVT alters the salience of species-typical frequency modulation in interactions among male túngara frogs. We presented males with a "conspecific" whine stimulus and two "non-conspecific" stimuli that differed from the whine in frequency modulation. We recorded their responses before and after treatment with AVT. We used avoidance of call overlap as evidence that a male recognizes a stimulus as salient, and chuck production as evidence of his competitive response to a proximate rival. We used call rate, whine frequency, and whine duration as additional measures of motivation to call and the motor production of advertisement calls. Following from previous studies, we predicted that (1) males are selective and respond to conspecific whines with less call overlap and more chucks than when presented with non-conspecific stimuli and (2) males alter vocal production and produce more chucks after treatment with exogenous AVT. We do not know, however, how AVT alters the salience of frequency modulation in recognizing and responding to conspecific rivals. If AVT tunes the processing of sensory stimuli, we predict (3) that there is an interaction between the effects of stimulus and AVT such that selective responses to conspecific signals are altered following treatment with AVT.

2. Methods

We conducted this study between June and August 2008 in Gamboa, Republic of Panama. We collected calling male túngara frogs in the area of Gamboa and tested them in laboratory facilities of the Smithsonian Tropical Research Institute (STRI). We tested males on the night of or the night after capture and released them at the site of capture after testing. Prior to release, we weighed and measured the snout-vent length of males and gave them a unique toe-clip combination to prevent re-testing. Our toe-clipping procedure followed the Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research compiled by the American Society of Ichthyologists and Herpetologists (available at https://asih.org). Toe-clipping has no discernable effect on túngara frogs, and we routinely recapture previously marked frogs.

2.1. Dynamic playback experiments

We conducted dynamic playback experiments following methods described in Goutte et al. (2010). A computer responded to a focal male's call, allowing for a more realistic volley of stimulus and response between the male and his simulated rival. Stimulus presentation and data collection was controlled with Túngara Evoked Call with Simultaneous Interaction Software (TECSIS), which was written for the purposes of interactive playback with túngara frogs and is described in detail in Goutte et al. (2010).

We placed each male in an acoustically transparent plastic bag with approximately 150 ml of rainwater. We inflated and closed the bags, then positioned them on a ledge in a darkened sound attenuation chamber so that males could partially emerge from the standing water. There were eight sound attenuation chambers, each of which measured 30.5 cm wide x 46.0 cm deep x 30.5 cm high. Each chamber was equipped with a microphone (Radio Shack, Electret model 33-3013) and a speaker (Cambridge Soundworks) connected to a computer via a multichannel data acquisition device (National Instruments USB-6221 Multifunction DAQ). Speakers were amplified with a 10 W Realistic SA-10 stereo amplifier.

At approximately 2000 h, we closed the sound attenuating chambers containing males. A 60 s loop of a recorded male túngara frog chorus was broadcast to all chambers at approximately 82 dB SPL (re 20 μPa) measured at the location of the focal male. We tested males only if they responded to this chorus. When a male began to call, we stopped the chorus stimulus and began testing. TECSIS evaluated incoming signals to confirm that they were a túngara frog call and responded with a stimulus. Computer responses followed the beginning of each male call by 1.3 s (the shortest time possible given processing constraints). If a male ceased calling for 150 s, the chorus stimulus was reinitiated until the male called again. This male call and computer response interaction continued until 25 calls were recorded. Upon reaching 25 calls, the male was tested with another stimulus.

We used three different synthetic stimuli for this study (Fig. 2). Our "conspecific" stimulus was a frequency- and amplitude modulated signal with the characteristics of túngara frog whine (Ryan and Rand, 1999), sweeping from 900 to 500 Hz over 370 ms. Two "non-conspecific" stimuli were a 700 Hz tone and a reverse frequency sweep with the same duration and amplitude envelope of the whine. The three stimuli thus differed only in frequency modulation, a characteristic that túngara frogs use as a cue for recognizing conspecific signals (Ryan, 1983; Wilczynski et al., 1995; Zelick et al., 1991). We created stimuli with a program made available by J.J. Schwartz. Only one stimulus was played to the frog during each playback period of 25 calls. We randomized the order in which the three stimuli were presented among males.

After testing males with the three stimuli in baseline experiments, we injected them subcutaneously with either arginine vasotocin (AVT, Sigma-Aldrich, 25 μg in 25 μl saline, $n = 25$ males) or a saline control (25 μl saline, $n = 5$ males) (see Kime et al., 2007 for dose-response analysis). We waited 30 min, then tested males again with the conspecific whine and the two non-conspecific stimuli. Males were similarly handled before and after treatment except for the injection.

We ceased all testing by 0200–0300 h. We left boxes open during the day to expose males that would be tested on the following night to a natural photoperiod. We used data only if a male completed all six playback periods in the same night. Eighteen of the 25 AVT-treated and four of the five saline-treated males completed testing in all six playback periods on either the night of capture or the following night.

2.2. Call analysis

The TECSIS computer program recorded a .wav sound file (sampling rate = 10 kHz) during each playback period that included both the computer stimulus and túngara frog calls. We used oscillograms generated in Adobe Audition to collect data on call timing within an entire playback period. We recorded the elapsed time at the beginning of each TECSIS stimulus and the elapsed time at the beginning of each focal male call. We then used custom MATLAB algorithms to analyze the first 20 calls from each male (excluding the first call that initiated TECSIS stimuli) (see also Kime et al., 2010). We first manually identified the whines and chucks in each recording. We used the following criteria to identify facultative chucks following whines: 1) a visible rise in amplitude following the whine and suggesting an increase in air flow (as observed in the oscillogram), 2) the presence of subharmonics in the chuck (identified from a spectrogram (512 point FFT length, 51 ms sliding Hamming window with 90% overlap)), and 3) auditory confirmation of the presence of a chuck. A second observer confirmed all chucks. For each identified whine and chuck, MATLAB batch processing algorithms measured spectral and temporal call parameters. Temporal parameters were measured from a rectified and smoothed (8 ms) amplitude envelope created from the oscillogram. Spectral parameters were measured from the spectrogram, from a frequency contour drawn through the fundamental of the túngara frog whine. In this paper, we report on eight variables described below.

We calculated three measures of call timing. Túngara frog males avoid overlapping the calls of neighboring males and generally do not call within 600 ms of the initiation of a conspecific call (Snedden and Rand unpublished data, cited in Greenfield and Rand, 2001). We calculated the proportion of a focal male's calls that were initiated <600 ms after the preceding TECSIS stimulus and took fewer such calls to suggest that a male was attending to and interacting with the stimulus. Túngara frog males generally call at a rhythmic rate of one call/two seconds (Ryan, 1985) but avoiding overlap with a rival might lengthen inter-call intervals. We thus calculated the median inter-call interval in each playback period as a second indicator that a male was attending to and interacting with the stimulus. We calculated the median inter-call interval rather than the mean to minimize the effect of infrequent pauses in calling. We also calculated the overall call rate for each playback period as an indicator of calling effort.

We report three measures of chuck production. We calculated the proportion of a focal male's calls with identified chucks in each of six the playback periods. We took this to be an indicator of a male's intention to produce chucks in response to a stimulus, regardless of the amplitude of those chucks. In a previous study (Kime et al., 2010), we observed that male túngara frogs treated with AVT in the field tend to produce low-amplitude chucks. In this study, we quantified the effect of AVT on chuck amplitude. We calculated the maximum amplitude of each chuck relative to the maximum amplitude of the preceding whine. We report the average relative amplitude of first chucks in each of the six playback periods. Phonotaxis experiments demonstrate that females prefer a whine-chuck to a whine only when the relative amplitude of the chuck is at least 50% that of the preceding whine (Baugh and Ryan, 2011). We therefore also calculated the mean number of "attractive" high-amplitude chucks per call in each of the six playback periods for each male.

In a previous study, males treated with AVT changed the characteristics of their whine (Kime et al., 2010). They produced shorter whines with higher frequencies, suggesting more vigorous calling (sensu Chu et al., 1998) as males push air through their vocal system. This may in turn compromise the production of attractive chucks. Túngara frogs

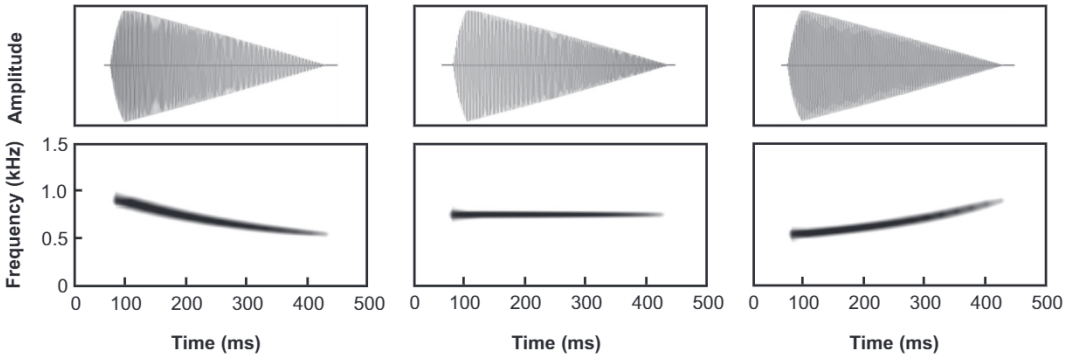

Fig. 2. Oscillograms (top) and spectrograms (bottom) of the conspecific whine, tone, and reverse-whine stimuli used in dynamic playback experiments.

do not inspire between the whine and chuck (Dudley and Rand, 1991). Males might deplete available air in their lungs during vigorous whines resulting in fewer or low amplitude chucks (see also Pauly et al., 2006). To confirm our previous findings regarding the effects of AVT on whines, we measured the duration and initial frequency of whines in each playback period. We measured the duration of whines from 90% maximum amplitude on the rise portion of the whine to 10% maximum amplitude on its fall (see Fig. 1). We measured the initial frequency of the whine at the point where the call was at 90% of its maximum amplitude. Males occasionally produce short prefixes prior to their whines (see Fig. 1); in measuring initial frequency at 90% of maximum amplitude, our batch processing algorithm ignored these prefixes.

2.3. Statistical analysis

We used SPSS for statistical analysis. We used a two-way repeated measures analysis of variance (ANOVA) to test for differences among playback periods in call timing, chuck production, and whine characteristics. We only included data from males who had sufficient data from all six playback periods. $N = 18$ of 25 males called in all six playback periods and were used for analysis of whines and chucks. $N = 7$ of those males called continuously without reinitiating the chorus and were used in analysis of call timing. We arcsine transformed data on the proportion calls overlapping the stimulus and the proportion of calls with chucks prior to statistical analysis. We evaluated the assumptions of ANOVA for all eight measurements and found few notable violations; we applied Greenhouse-Geisser correction to the F and P values of two tests (proportion of overlapping calls and chuck amplitude). We used an omnibus ANOVA to test for an effect of stimulus (prediction 1), an effect of AVT treatment (prediction 2), and an interaction between stimulus and treatment (i.e. whether treatment with AVT influences a male's selective response to whines, tones, and reverse-whines (prediction 3)). We used orthogonal planned contrasts to compare a male's vocal behavior during presentation of the conspecific whine to his behavior during presentation of the tone and reverse-whine, before and after treatment with AVT. Effect sizes are reported as partial eta^2 (η_p^2).

We compared changes in vocal behavior in males treated with AVT to changes in vocal behavior in males treated with a saline control. $N = 4$ saline-injected males called in all six playback periods, but only one produced 20 consecutive calls without re-initiation of the chorus stimulus. We used a repeated measures ANOVA in which the within subjects factor was treatment (pre- versus post-injection) and the between subjects factor was treatment group (AVT or saline). We included only the whine stimulus in statistical analysis, in order to focus on the effects of AVT versus saline while controlling for stimulus. We report the treatment * group interaction term of the ANOVA for variables in which we observed a significant effect of AVT treatment and for which there were data from N = 4 saline males.

3. Results

3.1. Call timing

We analyzed call timing data for $n = 7$ males who produced at least 20 consecutive calls without an intervening chorus during all six stimulus presentations. Males avoided overlapping stimulus whines; fewer than 5% of calls were initiated within 600 ms of the beginning of the whine stimulus. In a two-way repeated measures ANOVA, call overlap differed among stimuli ($F_{2,12} = 21.135$, $P = 0.002$, $\eta_p^2 = 0.779$). Call overlap did not change after treatment with AVT ($F_{1,6} = 1.240$, $P = 0.308$, $\eta_p^2 = 0.171$). The interaction between the main effects of stimulus and treatment was not statistically significant ($F_{2,12} = 0.427$, $P = 0.560$, $\eta_p^2 = 0.066$). Orthogonal planned contrasts showed that males overlapped whines less than tones and reverse whines both before and after treatment with AVT (pre-treatment: $F_{1,6} = 13.642$, $P = 0.010$, $\eta_p^2 = 0.695$; AVT-treated: $F_{1,6} = 16.771$, $P = 0.006$, $\eta_p^2 = 0.737$) (Table 1, Fig. 3a).

Table 1
Differences in vocal behavior across conditions for AVT-treated males. A two-way repeated measures analysis of variance tested the omnibus effects of stimulus, treatment with AVT, and the interaction between stimulus and treatment. Orthogonal planned contrasts tested for differences between conspecific whines and the two non-conspecific stimuli, before and after treatment with AVT. Effect sizes are partial eta squared (η_p^2). Significant effects are bolded.

	Stimulus	AVT	Interaction	Whine vs non-conspecific pre-injection	Whine vs non-conspecific AVT-treated
Overlapping calls	**$F_{2,12}$ = 21.135 η_p^2 = 0.779 P = 0.002**	$F_{1,6}$ = 1.240 η_p^2 = 0.171 P = 0.308	$F_{2,12}$ = 0.427 η_p^2 = 0.066 P = 0.560	**$F_{1,6}$ = 13.642 η_p^2 = 0.695 P = 0.010**	**$F_{1,6}$ = 16.771 η_p^2 = 0.737 P = 0.006**
Inter-call interval	**$F_{2,12}$ = 9.245 η_p^2 = 0.606 P = 0.009**	$F_{1,6}$ = 0.549 η_p^2 = 0.478 P = 0.058	$F_{2,12}$ = 0.133 η_p^2 = 0.022 P = 0.853	$F_{1,6}$ = 3.844 η_p^2 = 0.390 P = 0.098	**$F_{1,6}$ = 35.235 η_p^2 = 0.854 P = 0.001**
Calls per minute	$F_{2,12}$ = 0.031 η_p^2 = 0.005 P = 0.930	**$F_{1,6}$ = 20.013 η_p^2 = 0.769 P = 0.004**	$F_{2,12}$ = 2.847 η_p^2 = 0.322 P = 0.098	$F_{1,6}$ = 1.682 η_p^2 = 0.219 P = 0.242	$F_{1,6}$ = 2.101 η_p^2 = 0.259 P = 0.197
Proportion of calls with chucks	**$F_{2,34}$ = 0.919 η_p^2 = 0.351 P = 0.002**	$F_{1,17}$ = 2.697 η_p^2 = 0.137 P = 0.119	$F_{2,34}$ = 1.195 η_p^2 = 0.066 P = 0.314	**$F_{1,17}$ = 11.259 η_p2 = 0.398 P = 0.004**	$F_{1,17}$ = 3.305 η_p^2 = 0.163 P = 0.087
Chuck amplitude	**$F_{2,22}$ = 10.629 η_p^2 = 0.491 P = 0.001**	**$F_{1,11}$ = 17.926 η_p^2 = 0.620 P = 0.001**	$F_{2,22}$ = 0.971 η_p^2 = 0.081 P = 0.390	**$F_{1,11}$ = 8.708 η_p^2 = 0.442 P = 0.013**	**$F_{x1,11}$ = 4.797 η_p^2 = 0.304 P = 0.051**
Attractive chucks	**$F_{2,34}$ = 15.710 η_p^2 = 0.480 P < 0.001**	**$F_{1,17}$ = 14.332 η_p^2 = 0.457 P = 0.001**	**$F_{2,34}$ = 6.041 η_p^2 = 0.262 P = 0.008**	**$F_{1,17}$ = 23.353 η_p^2 = 0.579 P < 0.001**	$F_{1,17}$ = 2.568 η_p^2 = 0.131 P = 0.127
Whine duration	$F_{2,34}$ = 1.616 η_p^2 = 0.087 P = 0.220	**$F_{1,17}$ = 6.768 η_p^2 = 0.285 P = 0.019**	$F_{2,34}$ = 0.236 η_p^2 = 0.014 P = 0.734	$F_{1,17}$ = 0.275 η_p^2 = 0.016 P = 0.607	$F_{1,17}$ = 1.345 η_p^2 = 0.073 P = 0.262
Whine frequency	$F_{2,34}$ = 0.835 η_p^2 = 0.047 P = 0.433	**$F_{1,17}$ = 8.616 η_p^2 = 0.336 P = 0.009**	$F_{2,34}$ = 1.761 η_p^2 = 0.094 P = 0.192	$F_{1,17}$ = 0.461 η_p^2 = 0.026 P = 0.506	$F_{1,17}$ = 0.897 η_p^2 = 0.050 P = 0.357

When males avoid overlapping a stimulus, delays in calling may increase the median inter-call interval beyond the rhythmic one call per 2 s that is typical for this species. In a two-way repeated measures ANOVA, there was a significant difference in median inter-call interval among stimuli ($F_{2,12} = 9.245$, $P = 0.009$, $\eta_p^2 = 0.606$). Inter-call interval did not change after treatment with AVT ($F_{1,6} = 0.549$, $P = 0.058$, $\eta_p^2 = 0.478$). The interaction between the effects of stimulus and treatment was not statistically significant ($F_{2,12} = 0.133$, $P = 0.853$, $\eta_p^2 = 0.022$). Planned contrasts showed that males produced calls at significantly

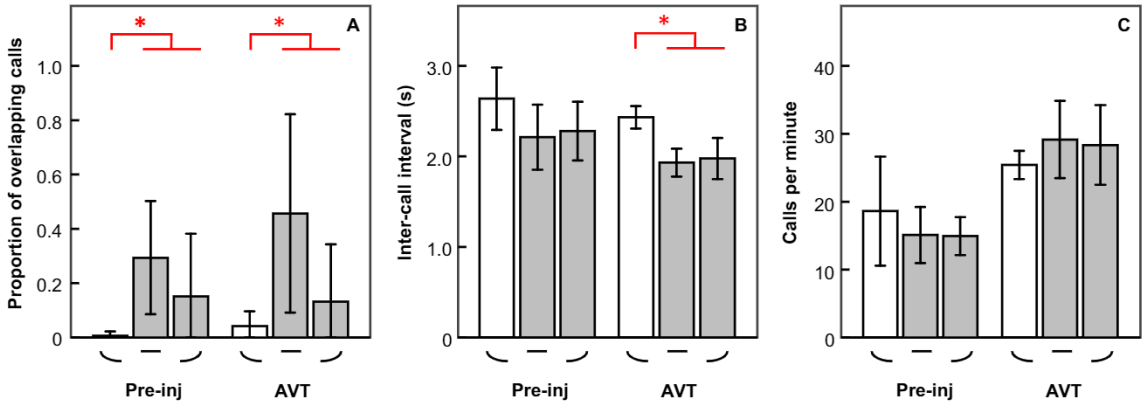

Fig. 3. Timing of male túngara frog calls when presented with whines, tones, and reverse-whines. (A) proportion of calls overlapping the stimulus, (B) median inter-call interval (one call per 2 s is typical of this species), (C) overall call rate. Bars show the mean among males ±95% CI. White bars = responses to the conspecific whine; dark bars = responses to the non-conspecific tone (left) and reverse-whine (right). Stars indicate a significant difference between the whine stimulus and the two non-conspecific stimuli in orthogonal planned contrasts. $N = 7$ males who produced at least 20 consecutive calls in all six trials.

longer median inter-call intervals in response to whines than to non-conspecific stimuli after treatment with AVT (pre-treatment: $F_{1,6} = 3.844$, $P = 0.098$, $\eta_p^2 = 0.390$; AVT-treated: $F_{1,6} = 35.235$, $P = 0.001$, $\eta_p^2 = 0.854$) (Table 1, Fig. 3b).

Overall call rate (calls per minute) did not differ among stimuli in a two-way repeated measures ANOVA ($F_{2,12} = 0.031$, $P = 0.930$, $\eta_p^2 = 0.005$). Males increased overall call rate following injection with AVT, suggesting fewer pauses in calling ($F_{1,6} = 20.013$, $P = 0.004$, $\eta_p^2 = 0.769$). The interaction between stimulus and AVT treatment was not significant ($F_{2,12} = 2.847$, $P = 0.098$, $\eta_p^2 = 0.322$). In planned contrasts, there was no significant difference in call rate between playback periods where males were presented with whines and non-conspecific stimuli, either before or after treatment with AVT (pre-treatment: $F_{1,6} = 1.682$, $P = 0.242$, $\eta_p^2 = 0.219$; AVT-treated: $F_{1,6} = 2.101$, $P = 0.197$, $\eta_p^2 = 0.259$) (Table 1, Fig. 3c).

3.2. Chuck production

We used the proportion of calls with chucks to assess a focal male's competitive response to stimuli before and after treatment with AVT. $N = 18$ males called in all six playback periods. In a two-way repeated measures ANOVA, there was a significant difference among stimuli in the proportion of calls with chucks ($F_{2,34} = 0.919$, $P = 0.002$, $\eta_p^2 = 0.351$). The proportion of calls with chucks did not change after treatment with AVT ($F_{1,17} = 2.697$, $P = 0.119$, $\eta_p^2 = 0.137$). The interaction between stimulus and AVT treatment was not statistically significant ($F_{2,34} = 1.195$, $P = 0.314$, $\eta_p^2 = 0.0.066$). Planned contrasts showed that males produced more calls with chucks in response to the whine stimulus than when presented with non-conspecific stimuli before injection with AVT (pre-treatment: $F_{1,17} = 11.259$, $P = 0.004$, $\eta_p^2 = 0.398$; AVT-treated: $F_{1,17} = 3.305$, $P = 0.087$, $\eta_p^2 = 0.163$) (Table 1, Fig. 4a).

Male túngara frogs might respond to appropriate stimuli by increasing not only the number of chucks, but also the amplitude (effort) with which they produce them. Conversely, overproduction of the whine might compromise a male's ability to produce high amplitude chucks. $N = 12$ males produced at least one chuck in all six playback periods and were included in this analysis. In a two-way repeated measures ANOVA, there was a significant effect of stimulus ($F_{2,22} = 10.629$, $P = 0.001$, $\eta_p^2 = 0.491$) and a significant effect of AVT treatment ($F_{1,11} = 17.926$, $P = 0.001$, $\eta_p^2 = 0.620$) on the amplitude with which males produced their first chuck. The interaction between the effects of stimulus and AVT treatment was not statistically significant ($F_{2,22} = 0.971$, $P = 0.390$, $\eta_p^2 = 0.081$). Prior to treatment with AVT, the amplitude of a male's first chuck was higher than that of the preceding whine (for the whine stimulus, $\bar{X}$ ratio of chuck to whine amplitude = 1.49 ± 0.54 SD). Chucks produced in response to whines had significantly higher relative amplitude than chucks produced in response to non-conspecific calls

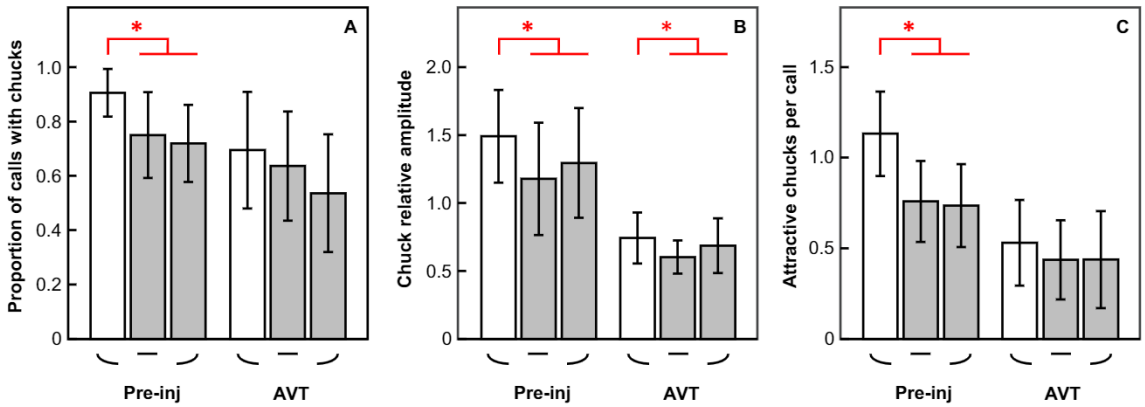

Fig. 4. Chuck production in male túngara frogs when presented with whines, tones, and reverse-whines. (A) proportion of calls with chucks, (B) amplitude of first chucks relative to the whine, (C) number of attractive high-amplitude chucks per call. Bars show the mean among males ±95% CI. White bars = responses to the conspecific whine; dark bars = responses to the non-conspecific tone (left) and reverse-whine (right). Stars indicate a significant difference between the whine and the two non-conspecific stimuli in orthogonal planned contrasts. (A, C) $N = 18$ males who produced calls in all six trials. (B) $N = 12$ males who produced chucks in all six trials.

($F_{1,11} = 8.708$, $P = 0.013$, $\eta_p^2 = 0.442$). After treatment with AVT, the relative amplitude of chucks decreased (for the whine stimulus, $\bar{X}$ ratio of chuck to whine amplitude = 0.74 ± 0.30 SD). Chucks produced in response to whines continued to have significantly higher relative amplitude than chucks produced in response to non-conspecific calls ($F_{1,11} = 4.797$, $P = 0.051$, $\eta_p^2 = 0.304$) (Table 1, Figs. 4b, 5).

Female túngara frogs show preferences for whine-chucks over whines only when the chuck is of sufficient amplitude, with a peak amplitude at least half that of the whine. In a two-way repeated measures ANOVA using data from $n = 18$ males who produced calls in all six trials, there was a significant effect of stimulus ($F_{2,34} = 15.710$, $P < 0.001$, $\eta_p^2 = 0.480$) and a significant effect of AVT treatment ($F_{1,17} = 14.332$, $P = 0.001$, $\eta_p^2 = 0.457$) on the production of attractive chucks. The interaction between main effects of stimulus and AVT was also significant ($F_{2,34} = 6.041$, $P = 0.008$, $\eta_p^2 = 0.262$). Prior to treatment with AVT, individual males produced more high amplitude "attractive" chucks per call in response to whines than in response to non-conspecific stimuli ($F_{1,17} = 23.353$, $P < 0.001$, $\eta_p^2 = 0.579$). Males decreased the number of attractive chucks following treatment with AVT. The number of attractive chucks did not differ between whines and non-conspecific stimuli after treatment with AVT ($F_{1,17} = 2.568$, $P = 0.127$, $\eta_p^2 = 0.131$) (Table 1, Fig. 4c, Fig. 5).

3.3. Whine characteristics

We analyzed characteristics of the whine to confirm results from a prior field study (Kime et al., 2010) regarding the effects of AVT on whine production. $N = 18$ males produced whines in all six playback periods. There was no difference among stimuli in the duration or initial frequency of a male's whine (whine duration: $F_{2,34} = 1.616$, $P = 0.220$, $\eta_p^2 = 0.087$; whine frequency: $F_{2,34} = 0.835$, $P = 0.433$, $\eta_p^2 = 0.047$). Males produced shorter whines with higher initial frequency after treatment with AVT (whine duration: $F_{1,17} = 6.768$, $P = 0.019$, $\eta_p^2 = 0.285$; whine frequency: $F_{1,17} = 8.616$, $P = 0.009$, $\eta_p^2 = 0.336$). There was no significant interaction between the main effects of stimulus and AVT (whine duration: $F_{2,34} = 0.236$, $P = 0.734$, $\eta_p^2 = 0.014$; whine

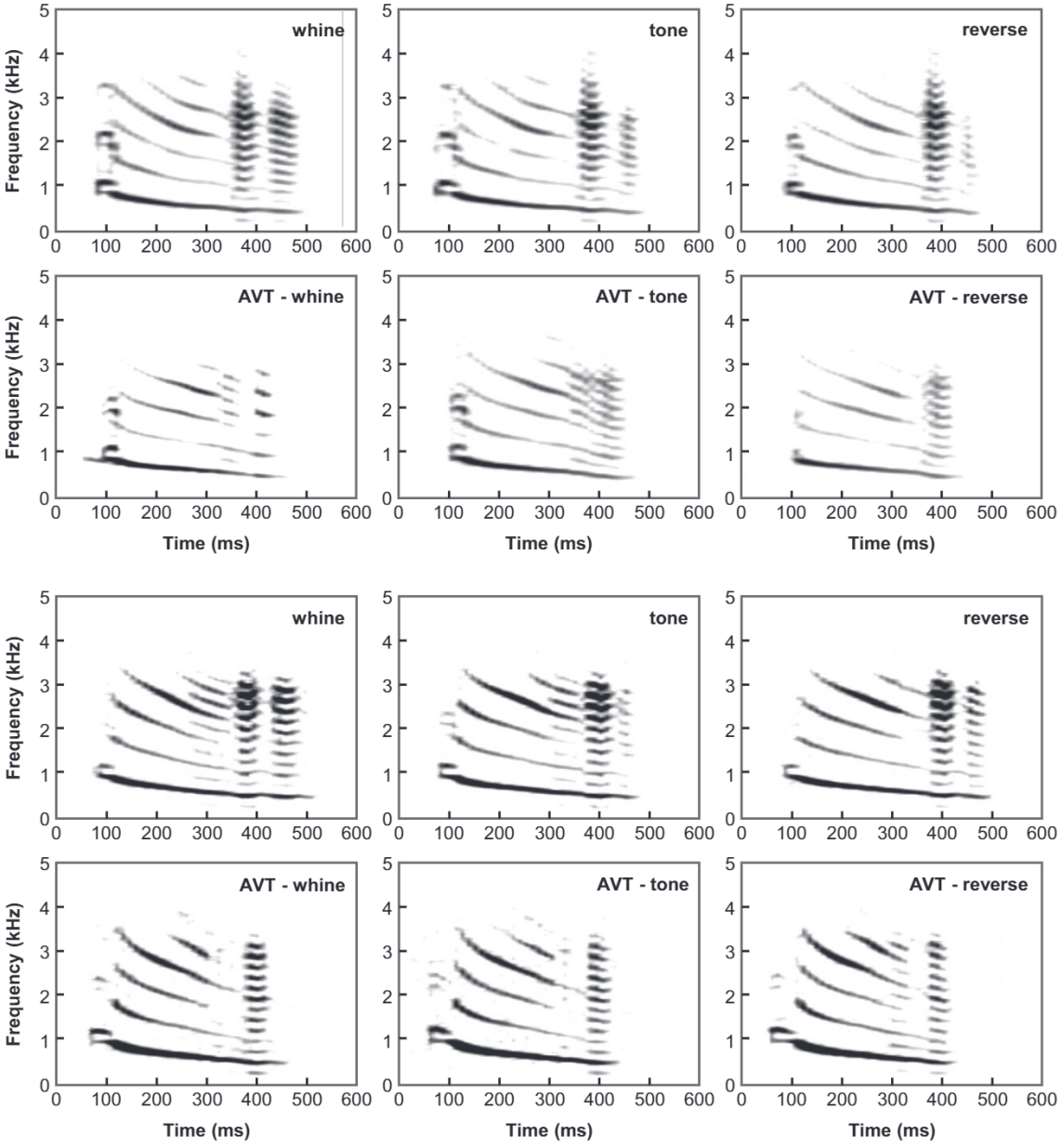

Fig. 5. Spectrograms showing sample calls from two males. Responses to whines, tones and reverse whines, before and after treatment with AVT.

frequency: $F_{2,34} = 1.761$, $P = 0.192$, $\eta_p^2 = 0.094$). In planned contrasts, there was no significant difference in whine duration or frequency between playback periods where males were presented with whines and non-conspecific stimuli, either before or after treatment with AVT (pre-treatment whine duration: $F_{1,17} = 0.275$, $P = 0.607$, $\eta_p^2 = 0.016$; pre-treatment whine initial frequency: $F_{1,17} = 0.461$, $P = 0.506$, $\eta_p^2 = 0.026$; AVT-treated whine duration: $F_{1,17} = 1.345$, $P = 0.262$, $\eta_p^2 = 0.073$; AVT-treated whine initial frequency: $F_{1,17} = 0.897$, $P = 0.357$, $\eta_p^2 = 0.050$; AVT) (Table 1, Fig. 5).

3.4. Saline controls

We used a repeated measures ANOVA in which the within subjects factor was treatment (pre- versus post-injection) and the between subjects factor was treatment group (AVT or saline) to compare changes in vocal behavior after treatment with AVT to changes in vocal behavior after a control injection with saline. We included only the whine stimulus in our statistical analysis, in order to focus on the effects of AVT versus saline while controlling for stimulus. We report the treatment * group interaction term of this analysis for four call characteristics that showed significant changes in AVT-treated males and for which there was sufficient data from saline males: chuck relative amplitude, number of attractive chucks per call, whine duration, and whine frequency. Fig. 6 shows within-male changes in these four call characteristics for all three stimuli; only the whine stimulus is included in the statistical analysis below.

The observed decrease in chuck relative amplitude following treatment with AVT was significantly different than the effect of a control injection with saline (repeated measures ANOVA treatment * group interaction term, $F_{1,14} = 9.102$, $P = 0.010$, $\eta_p^2 = 0.392$). The effects of AVT on the number of attractive chucks per call, whine duration, and whine frequency were not significantly different than the effects of a saline control (attractive chucks: $F_{1,20} = 3.171$, $P = 0.090$, $\eta_p^2 = 0.137$; whine duration: $F_{1,20} = 1.047$, $P = 0.319$, $\eta_p^2 = 0.050$; whine frequency: $F_{1,20} = 0.801$, $P = 0.381$, $\eta_p^2 = 0.039$) (Fig. 6).

4. Discussion

We used an interactive playback paradigm to investigate whether arginine vasotocin (AVT) alters the salience of species-typical frequency modulation in interactions among male túngara frogs. Our data are consistent with the hypothesis that arginine vasotocin (AVT) modulates vocal behavior by influencing the general motivation to call and the motor production of calls. As in a previous study (Kime et al., 2010), exogenous treatment with AVT increased call rate and caused changes in whines and chucks that likely reflect changes in air flow through the vocal system. We did not, however, find evidence that AVT alters the selectivity with which males respond to conspecific and altered signals. Male túngara frogs were less likely to overlap calls and were more likely to produce chucks in response to stimuli with species-typical frequency modulation than in response to two non-conspecific stimuli. This selective response did not change after treatment with AVT.

We predicted that (1) male túngara frogs would respond selectively to stimuli with species-typical frequency modulation. Many frogs avoid overlapping acoustic signals, either because males cannot listen while calling or because females disfavor lagging calls (Grafe, 1996; Greenfield et al., 1997; Höbel, 2015; Schwartz, 1987; Schwartz and Rand, 1991). In túngara frogs, males inhibit calling following another conspecific male (Greenfield and Rand, 2001) and females prefer leading to following calls if the following call is produced within 250 ms of the end of the leader call (Ryan, unpublished data). In the current study, males avoided overlapping their calls with a conspecific whine stimulus more than non-conspecific tones and reverse-whines, suggesting that they differentially attend to and interact with a stimulus that has species-typical frequency modulation.

Males also show a more competitive response to conspecific signals

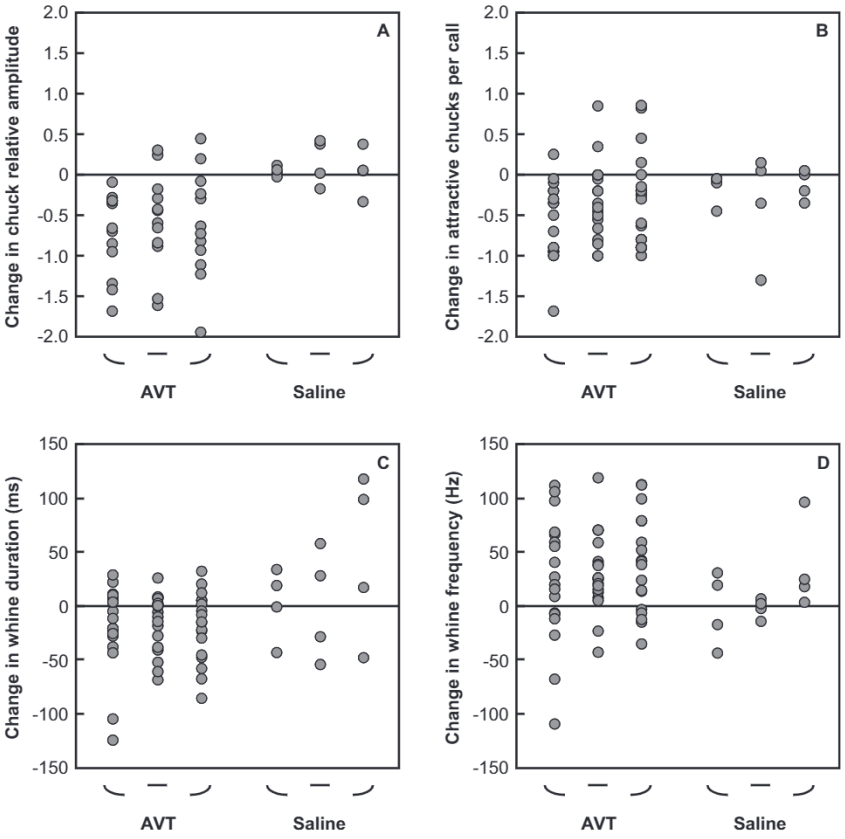

Fig. 6. Changes in the vocal behavior of individual males after treatment with AVT or a saline control, in response to a conspecific whine, tone, or reverse-whine stimulus. (A) change in amplitude of first chucks relative to the whine, (B) change in number of attractive high-amplitude chucks per call, (C) change in whine duration, (D) change in whine frequency. Each point for each stimulus represents one male. (A) N = 12 AVT males and $N = 4$ saline males who produced chucks in all six trials. (B, C, D) N = 18 AVT males and N = 4 saline males who produced calls in all six trials.

than non-conspecific signals. Previous studies showed that males produce more chucks in response to conspecific whines than non-conspecific reverse-whines (Ryan, 1983; Zelick et al., 1991) and that females fail to respond with phonotaxis to reverse-whines (Wilczynski et al., 1995). In our dynamic playback experiment, males were similarly more likely to produce chucks in response to whines than non-conspecific stimuli. They also produced these chucks at higher amplitude relative to the preceding stimulus when the stimulus was a whine. Males thus produced more attractive high-amplitude chucks in response to whines than non-conspecific stimuli. These data on call overlap avoidance and chuck production add to the long-standing conclusion that frequency modulation is important for species recognition in túngara frogs.

We also predicted that (2) males would alter vocal production and produce more chucks after treatment with exogenous AVT. Three lines of evidence from the current study support the idea that AVT influences motivation to call and vocal production in túngara frog males. Males increased call rate, decreased the duration of their whines, and increased the frequency of their whines after treatment with AVT. The observed increase in call rate is consistent with results from túngara frogs and other species that are more likely to resume calling, and call more, after treatment with AVT (e.g. Boyd, 1994; Burmeister et al., 2001; Kime et al., 2007; Ten Eyck, 2005). Likewise, shorter whines may reflect more vigorous calling (sensu Chu et al., 1998) and altered air flow during the whine (Kime et al., 2010). Male túngara frogs are able to modulate the amplitude of their whines and chucks in response to increasing background noise and simulated rivals (the Lombard effect) (Halfwerk et al., 2017). They presumably do so by increasing air flow from the lungs (Dudley and Rand, 1991; Kime et al., 2019, 2013), which can result in shorter calls if males exhaust the limited air in their lungs (Pauly et al., 2006). The increase in initial call frequency following treatment with AVT also suggests that males increase air flow early in the call (Kime et al., 2019).

An increase in air flow during the whine may influence the production of subsequent chucks. Males decreased the amplitude of first chucks after treatment with AVT, resulting in fewer “attractive” chucks per call. The current analysis quantifies an earlier observation from Kime et al. (2010), which reported low-amplitude chucks in AVT males and suggested that males treated with exogenous AVT are unable to produce attractive chucks because they over-exert in the production of whines. It is also similar to the effects of AVT on cricket frogs, where “vigorous” calling in AVT-treated males compromised an aggressive response (a decrease in dominant frequency) (Chu et al., 1998). In other words, we speculate that túngara frog males treated with exogenous AVT decrease the number of attractive chucks that they produce not because they are less motivated or choose not to produce them but instead because they overproduce the whine. Among the possible alternative hypotheses for low-amplitude chucks, prior research on vocal production in this species best supports the idea that they reflect a constraint on vocal production due to limited air in their vocal system (Dudley and Rand, 1991; Kime et al., 2019, 2010; Pauly et al., 2006).

The sample size of saline controls in the current study was insufficient to statistically confirm that changes in call rate, whines, and attractive chucks are attributed to AVT rather than injection per se, but the results of this and other studies strongly suggest that this is the case. In the current study, we saw no indication from visual inspection of within-individual call changes that saline treated controls substantially altered whine duration, whine frequency, or the production of attractive chucks (Fig. 6). Our results are also consistent with prior studies investigating the effects of AVT on anuran vocal behavior, which have demonstrated significant differences between males treated with AVT and saline controls. Several studies on túngara frogs and other species have shown that, compared to saline controls, AVT-treated males are more likely to call, call more, and produce calls with altered spectral and temporal call characteristics (e.g. Boyd, 1994; Chu et al., 1998; Kime et al., 2010, 2007; Marler et al., 1995; Miranda et al., 2015; Propper and Dixon, 1997; Ten Eyck, 2005; Tito et al., 1999; reviewed in Boyd, 2013; Wilczynski et al., 2017).

We did not find evidence that AVT changes the salience of frequency modulation in responding selectively to conspecific signals. If AVT alters which signals are recognized as socially appropriate, we predicted (3) an interaction between the effects of stimulus and AVT such that selective responses to the conspecific signal are altered following treatment with AVT. Regarding call overlap as a measure of signal recognition, we saw no such interaction. Males continued to avoid overlapping conspecific whines more than non-conspecific stimuli following treatment with AVT. The same was true regarding chuck production as a competitive response to conspecific males. In our study and others (Ryan, 1983; Zelick et al., 1991), untreated males were more likely to produce chucks in response to whines with species-typical frequency modulation. In the current study, there was no interaction between the effects of stimulus and AVT on the proportion of calls with chucks. The only variable where AVT differentially altered vocal behavior among stimuli was the number of attractive chucks per call, where AVT treated males showed a greater decrease in attractive chucks when responding to the whine stimulus. As discussed above, we suggest that this is due to a constraint on vocal production rather than altered recognition of the conspecific signal. Since males produced more attractive chucks in response to whines before injection, a constraint on their production following treatment with AVT would have a larger effect.

Our laboratory playback study thus confirms the results of field experiments in this species showing that AVT modulates the vocal behavior of male túngara frogs via its effects on motivation to call and motor production (Kime et al., 2010, 2007). Experiments in other anuran species also suggest that AVT affects motivation and motor production (reviewed in Boyd, 2013; Wilczynski et al., 2017). We did not, however, see a change in behavioral responses that would suggest that there was a change in the processing of the signals that we presented (as suggested for bullfrogs by Boyd, 1994). In frogs, recognition of conspecific signals likely begins at the level of the midbrain torus semicircularis (Wilczynski and Endepols, 2007). Cells in the torus respond selectively to behaviorally relevant combinations of tones (Feng et al., 1990) and in túngara frogs show higher immediate-early gene expression after playbacks with the conspecific whine (Hoke et al., 2004). In our study, AVT altered vocal behavior but did not change selective responses to species-typical stimuli. This is similar to previous work in green treefrogs, where AVT increased vocal production even though it decreased sensitivity of cells in the torus semicircularis of some animals (Penna et al., 1992).

Both male and female túngara frogs respond to conspecific calls, and frequency modulation is important for species recognition in both males and females (Ryan, 1983; Wilczynski et al., 1995). Males are generally more permissive than females in their responses to non-conspecific stimuli, as is expected considering the potential costs of a false alarm for females (Bernal et al., 2007). It is possible that such behavioral differences between males and females are related to sex differences in the AVT system (Boyd, 1997; Marler et al., 1999). However, exogenous treatment with AVT appears to increase motivation to mate in both male and female túngara frogs – for males to join a chorus and produce calls, and for females to perform phonotaxis to the male signal (Baugh and Ryan, 2017; Kime et al., 2007). Likewise, treatment with AVT also has little effect on the permissiveness of female túngara frogs to non-conspecific signals (Baugh and Ryan, 2017).

AVT modulates social behavior, but its specific effects vary among taxa (Goodson and Bass, 2001). Studies in some vertebrate groups suggest that AVT influences the processing of and responses to socially-appropriate stimuli (reviewed in Albers, 2012; Bester-Meredith et al., 2015; Rose and Moore, 2002; Wilczynski et al., 2017). This has yet to be clearly demonstrated in anurans. In this study, we investigated whether AVT influences the selectivity with which male túngara frogs respond to the calls of conspecific males. We confirmed previous work showing that AVT alters vocal behavior in male túngara frogs (Kime et al., 2010,

2007), but did not find evidence that it alters selective responses to conspecific versus non-conspecific signals. Studying the effects of AVT on male (and female) responses to within-species variation in whines and chucks may give more insight into how this hormone influences an individual's responses to social stimuli.

Declaration of competing interest

None.

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