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Arginine vasotocin induces calling behavior with a female social stimulus and interacts with gonadotropins to affect sexual behaviors in male *Xenopus tropicalis*

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HIGHLIGHTS

- Arginine vasotocin (AVT) induces calling behavior in male *X. tropicalis*.
- The presence of a female stimulates male calling regardless of AVT treatment.
- AVT and gonadotropins interact to influence calling and clasping behavior in males.

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ABSTRACT

Arginine vasotocin (AVT) and the mammalian homologue, arginine vasopressin (AVP), modulate vertebrate social behaviors, including vocalizations in male anurans. To study the impact of AVT and social stimuli on calling in male *Xenopus tropicalis*, we injected males with vehicle, 1 μg, or 10 μg AVT and recorded vocalizations under four social contexts (no stimulus, with male call playback, with a female, and with call playback and a female). More males called when injected with 10 μg AVT. Furthermore, calling males called only when paired with a female. We identified four call types: long fast trill; short fast trill; slow trill; or click. Next, we injected males with vehicle, 10 μg, or 20 μg AVT and recorded vocalizations with or without a female. AVT treatment did not affect calling in this experiment, but we confirmed that more males, regardless of AVT treatment, called when a female was present. Then we evaluated the effect of human chorionic gonadotropin (hCG) on male sexual behavior. 20 IU hCG elevated behavior compared to controls while the 10 IU hCG treatment group was not different from either treatment. Last, we examined the effect of AVT on hCG-induced reproductive behavior. Males were injected with 10 IU hCG or with 10 IU hCG and 20 μg AVT. Males receiving hCG and AVT clasped and called significantly more than males receiving hCG only. Our results suggest that AVT and a female stimulus induce vocalizations in a male pipid anuran, *X. tropicalis*, and the interaction between gonadotropins and neurohormones influences reproductive behaviors in this anuran amphibian.

1. Introduction

Arginine vasotocin (AVT), a peptide hormone in non-mammalian vertebrates, and arginine vasopressin (AVP), the mammalian homologue, modulate several social behaviors and many of the endocrine and neuroendocrine activities of AVT/AVP on behavior have been conserved throughout vertebrate evolution (reviewed by [1,18,20,45]). For example, AVT/AVP affects reproductive and aggressive behaviors in fishes [16], reptiles [15], birds [21], and mammals [4,37]. This neuropeptide system also influences bird flocking behavior [22] as well as mammalian pair-bonding [40] and parental care behavior [4]. Collectively, these studies demonstrate that the AVT/AVP system affects a variety of social behaviors across vertebrate clades.

In amphibians AVT treatment facilitates several mating behaviors. The first study investigating the effect of this neuroendocrine pathway on behavior found that AVT inhibits the release call in female leopard frogs, *Rana pipiens* [13]. Other studies in female anuran amphibians demonstrate that AVT treatment increases call phonotaxis in American bullfrogs, *Rana catesbeiana*, [5] and American toads, *Bufo americanus* [47]. In male urodele amphibians, AVT affects spermatophore discharge, pheromone release, and androgen-induced courtship tail vibration behavior in Japanese red-bellied newts, *Cynops pyrrhogaster*, [60] and AVT enhances amplectic clasping behavior in male roughskin newts, *Taricha granulosa* [38,39], while an antagonist

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to AVP or an anti-AVT immune serum inhibits this sexual behavior in this species [38]. These studies demonstrate that AVT affects various reproductive behaviors in male and female amphibians.

The most broadly established behavioral role in vertebrates, including anuran amphibians, for the AVT/AVP system is vocal modulation [20,65]. In anurans AVT administration facilitates the display of male calling behavior in several species including *R. catesbeiana* [5]; green treefrog, *Hyla cinerea*, [42]; gray treefrog, *Hyla versicolor* [49]; cricket frog, *Acris crepitans* [34]; American toad, *B. americanus* [48]; Great Plains toad, *Bufo cognatus* [44]; túngara frog, *Physalaemus pustulosus* [28]; and the Puerto Rican coquí frog, *Eleutherodactylus coqui* [52]. Collectively, these studies demonstrate that AVT is an important modulator of calling behavior in male anurans.

In addition to AVT, social conditions are also important in influencing anuran vocalizations. Natural or simulated call recordings are commonly used to stimulate calling behavior in AVT-treated males [5,8,10,42]. Boyd [5] did not observe spontaneous calling in AVT-treated male bullfrogs, but they called in response to a recording of calls from a male conspecific, and male cricket frogs treated with AVT called sooner and more vigorously during and after a simulated acoustic agonistic encounter [10]. In field experiments with Puerto Rican coquí frogs, satellite males began calling after an AVT injection and were also more likely to move into a new territory than AVT-injected territorial males [52]. Finally, in field experiments with gray treefrogs intruding males were more likely to call and take over a resident's calling site when treated with AVT [49] and proximity to another calling male influenced calling behavior in AVT-treated males [61]. Taken together, these studies suggest that AVT and social stimuli interact to affect vocalizations in male anurans.

Xenopus tropicalis has recently become a popular model organism. Male vocal behavior induced using gonadotropin has been reported previously [57,63]. Vigny [63] described male *X. tropicalis* advertisement calls as “very deep trills” varying from 1.5 s to 10 s in duration, and Tobias et al. [57] described these calls as having a single low frequency peak. However, the effect of AVT administration on calling in this species has not been studied, and to the best of our knowledge, there is no evidence that AVT induces calling behavior in the family Pipidae. Thus, the purpose of this study was to investigate whether exogenous AVT induces calling behavior in male *X. tropicalis* and whether different social stimuli influence male vocalizations. We evaluated how AVT treatment and different social stimuli, including exposure to a female and male call playback recording, influenced male vocalizations. We predicted that exogenous AVT would increase the probability for a male to call and this probability would also increase with increasing AVT dose. Next, we examined how AVT treatment and the presence or absence of females influenced male calling. Androgens modulate anuran brain AVT signaling and calling behavior [6,42], and these androgenic effects are influenced by social context [7]. Therefore, we then investigated whether hCG treatment, which increases circulating androgens and calling behavior in *Xenopus laevis* [64], facilitates sexual behavior in *X. tropicalis*. Finally, we examined the effect of AVT treatment on hCG-induced calling and clasping behavior to determine whether AVT interacts with gonadal activation of steroid production to influence reproductive behavior.

2. Methods

2.1. Animal husbandry

X. tropicalis were obtained from Xenopus Express (Brooksville, FL), Nasco (Modesto, CA), the Grainger laboratory (Robert Grainger, University of Virginia, Charlottesville, VA), or bred in the Propper laboratory (Catherine Propper, Northern Arizona University, Flagstaff, AZ) and housed at the Northern Arizona University Biological Sciences Annex. Frogs were housed in 40 L aquaria containing 6 L of salt-conditioned (0.3 g/L R/O Right, KENT Marine, Franklin, WI) reverse osmosis (RO) water. Aquaria were kept in a temperature controlled room maintained at 25 °C with a 12-hour light:dark photoperiod (0000 h:1200 h), and each aquarium housed one to six frogs of the same sex and source. Frog health was assessed daily. Ninety percent of the water was changed three times per week after which each frog was fed three pellets of Frog Brittle (Nasco). Northern Arizona IACUC (Protocol Number 09-006) approved all husbandry and experimental procedures.

2.2. General behavior trial methods

For all experiments, the following general methods were used. At 1100 h on the day of the experiment, each male was weighed and then placed in a clean aquarium with 6 L of salt-conditioned RO water. After weighing, hormone treatments were injected using 50 μL amphibian Ringer's solution (diluted 10-fold from stock solution: 114.99 mM NaCl, 2.52 mM $KHCO_3$, 0.973 mM $CaCl_2$) as a vehicle. Following the injection, frogs were returned to the experimental tank and allowed to acclimate. Tanks were separated with acoustic sound foam to help insulate individual males from calls of nearby males. All females used in behavior trials were uninjected sexually mature adults. In all behavior trials throughout this study, clasped females extended their hind limbs and swam until the male was dislodged indicating that they were sexually unreceptive [25,68]. This condition is known to induce advertisement calling in male *X. laevis* [26,64,70]. Behavior trials started at lights-out (1200 h) and frog behavior was observed under red light. Vocalizations from each male frog were recorded using an H2 hydrophone (Aquarian Audio Products, Anacortes, WA) submerged in the center of the aquarium that was attached to a Sony ICD-P Series digital recorder (Sony Corporation, New York, NY). Recordings were later analyzed using Raven Pro (Cornell Lab of Ornithology, Ithaca, NY) for the display of calling behavior. Each call bout was analyzed for duration and click rate (clicks/s), and the total time spent calling was determined.

2.3. Experiment 1: effect of AVT and social stimuli on calling and clasping behavior

Ten males (eight Xenopus Express, two Nasco) with a mean weight of 11.2 g (range: 7.3–15 g) were used to evaluate the effect of AVT and different social contexts on calling behavior in male *X. tropicalis*. Experiment 1 was conducted from late June to early July in 2008. Each frog was injected intraperitoneally (ip) with three different treatment dosages of AVT (Sigma-Aldrich cat # V0130, St. Louis, MO, USA). Frogs were injected with vehicle control, 1 μg AVT, and 10 μg AVT which are within the range of effective ip dosages previously used to induce vocalizations in other anuran species [5,30,44]. Each treatment injection was given on a different day in randomized order with four days in between treatment injections. On the day of each injection male vocalizations were recorded under four different social contexts which were presented in the following order: 1) male alone; 2) male exposed to call playback of conspecific male; 3) male paired with an untreated adult female; and 4) male paired with the female and exposed to call playback. Each context lasted 90 min. The order of social contexts presented remained the same for all frogs for the three behavior trial days. The playback recording consisted of previously recorded calls from an individual conspecific male that was played on a loop over a loudspeaker in the room. Each male was focally recorded for 15 min out of the 90 min for each social context [31]. The proportion of frogs calling in each AVT treatment and in each social context was determined. Each male was also focally observed under red light for 5 min for clasping behavior during social contexts when the female was present (contexts 3 and 4).

2.4. Experiment 2: effect of AVT and female stimulus on calling and clasping behavior

To determine whether male calling behavior in Experiment 1 was induced by the female social stimulus rather than the time after AVT

injection, we used male *X. tropicalis* (Grainger lab) with a mean weight of 8.5 g (range: 7–11 g) to evaluate the effect of a female stimulus on calling behavior in AVT-treated males. Experiment 2 was conducted during October of 2008. Each male was randomly assigned to one of three AVT treatment groups (n = 12). The ip AVT dosages 10 μg, 20 μg or vehicle were used to determine if there was a dose effect of AVT on male calling behavior. In order to determine whether females induced vocalizations in males, calling behavior was recorded under the sequentially introduced social contexts: 1) male alone; 2) male paired with a female; 3) female removed; and 4) female reintroduced. Each male was focally recorded for 30 min out of the 75 min for each social context. The amount of time each call type (described from Experiment 1; Table 1) was displayed, total time calling (excluding clicks), and the proportion of frogs calling in each AVT treatment and for each social stimulus were determined. Each male was also focally observed for clasping behavior as in Experiment 1.

2.5. Experiment 3: effect of hCG on calling and clasping behavior

Other studies have used hCG treatment to induce sexual behavior in male *X. tropicalis* [63] and *X. laevis* [26,55,64,70]. Experiment 3 was conducted in late May of 2010 using male *X. tropicalis* (Propper lab) with a mean weight of 10.8 g (range: 7.5 g–14.5 g) to determine a dose–response effect of hCG on sexual behavior. Each male was randomly assigned to one of four hCG treatment groups (n = 6). Frogs were injected in the dorsal lymph sac with one of the following: vehicle control, 5, 10, or 20 International Units (IU) of hCG (Sigma-Aldrich cat # CG10). A female was paired with the male at lights out and left in the tank for the duration of the experiment. Calling behavior was recorded for 5 h, and male clasping behavior was focally observed under red light for five min rotating increments totaling 20 min per hour over the course of 5 h (100 min total). The number of clasp attempts, amount of time that each call type (Table 1) was displayed, and total time calling (excluding clicks) was determined.

2.6. Experiment 4: effect of AVT and hCG on calling and clasping behavior

Experiment 4 was designed to determine whether the effect of AVT on male sexual behavior in *X. tropicalis* is influenced by co-treatment with gonadotropin. Experiment 4 was conducted from late June to early July of 2010 using male *X. tropicalis* (Grainger lab; mean weight = 9.2 g, range: 6.5 g–10.9 g). Based on results from Experiment 3, all males in this experiment received 10 IU hCG to induce a baseline sexually active state. Males received one of two treatment injections in the dorsal lymph sac (n = 12): 10 IU hCG (control treatment) or 10 IU hCG and 20 μg AVT. At lights out, a female was paired with the male and left in the tank for the duration of the experiment. Calling and clasping behaviors were evaluated as described in Experiment 3.

2.7. Statistics

For Experiments 1 and 2 a G-test was used to test for differences in the proportion of males observed calling and the proportion observed clasping among AVT treatments. A G-test was also used to test for differences in the proportion of calling males, regardless of AVT treatment, observed calling among social stimuli. For post-hoc analysis, Fisher's exact test was used to test for individual differences in the proportion of males calling between hormone treatments as well as between social stimuli. Data for time spent calling and clasp attempts in Experiments 2, 3 and 4 were not normally distributed. Therefore, we used a nonparametric Kruskal–Wallis test (Mann–Whitney U test for Experiment 4) followed by a post-hoc Mann–Whitney U test to compare the frequency of clasp attempts, total time calling, and time of individual call types (excluding clicks) among hormone treatment groups. In Experiment 4 Fisher's exact test was also used to test for differences between treatment groups in the proportion of males observed calling more than 100 s. All statistical analyses were conducted using JMP Version 9 (SAS, Cary, NC).

Table 1
Definition of calls displayed by *X. tropicalis* based on call parameters. Call spectrograms and waveforms are illustrated in Fig. 1.

Call name	Clicks/s	Call bout length
Fast long trill	>20	≥1 s
Fast short trill	>20	<1 s
Slow trill	5–20	≥5 clicks
Click	<5	<5 clicks

3. Results

3.1. Calling behavior in X. tropicalis

From Experiment 1 we identified and described four different types of calls displayed by *X. tropicalis* which were regularly displayed over the course of our study. Call types are based on call parameters including the call click rate and/or the length of the call bout. These call types include: 1) long fast trills; 2) short fast trills; 3) slow trills; and 4) clicks (Table 1; Fig. 1). A long fast trill is a call bout lasting at least 1 s and with a click rate of at least 20 clicks/s. A short fast trill is a call bout less than 1 s in length and a click rate of at least 20 clicks/s. A slow trill is a call bout with a click rate between 5 and 20 clicks/s. A trill (fast or slow) consists of at least 5 clicks in a call bout. Any call bout consisting of less than 5 clicks or with a click rate of less than 5 clicks/s was identified as a click. All four calls that we observed had a dominant frequency of approximately 600 Hz. The fast long trill that we observed is similar spectrally to the male *X. tropicalis* advertisement call previously described [57,63] and was the call most frequently displayed by males and was commonly produced before and during the time males also displayed clasping behavior (personal observation).

3.2. Experiment 1: effect of AVT and social stimuli on calling and clasping behavior

In Experiment 1 AVT significantly increased the proportion of males calling compared to controls ($G^2 = 6.01$, $P = 0.0495$; Fig. 2A). Post-hoc analysis demonstrated that 10 μg AVT induced calling compared to control animals (Fisher's exact, $P = 0.029$). All other comparisons did not differ ($P > 0.05$). Only one male in the control treatment was observed calling. Thus, we were unable to statistically analyze the data for time spent calling and time displaying individual call types. There was a significant difference in the proportion of frogs calling among social stimuli irrespective of AVT treatment ($G^2 = 31.43$, $P < 0.0001$; Fig. 2B). All calling males across all treatment groups called only during social contexts when the female was present in the tank. The proportion of calling males that called when paired with a female only was significantly different compared to when males were alone and compared to when males were exposed to the playback recording only (Fisher's exact, $P < 0.0001$ for each comparison). The proportion of males that called when paired with a female while also exposed to the playback recording was significantly different compared to when males were alone and compared to when males were exposed to the playback recording only (Fisher's exact, $P = 0.0325$ for each comparison). When males called, the duration (s) for each call type (mean ± SE, # callers) when paired with females (no playback recording) was: 10.4 ± 2.8, 6 for long fast trills, 4.5 ± 0.8, 4 for short fast trills, and 7.1 ± 1.3, 2 for slow trills and when paired with a female plus playback record was: 10.4 ± 9.4, 5 for long fast trills, 3.2 ± 1.2, 2 for short fast trills, and 46.8, 1 for slow trills. One male (1 μg AVT) was observed clasping among all treatment groups.

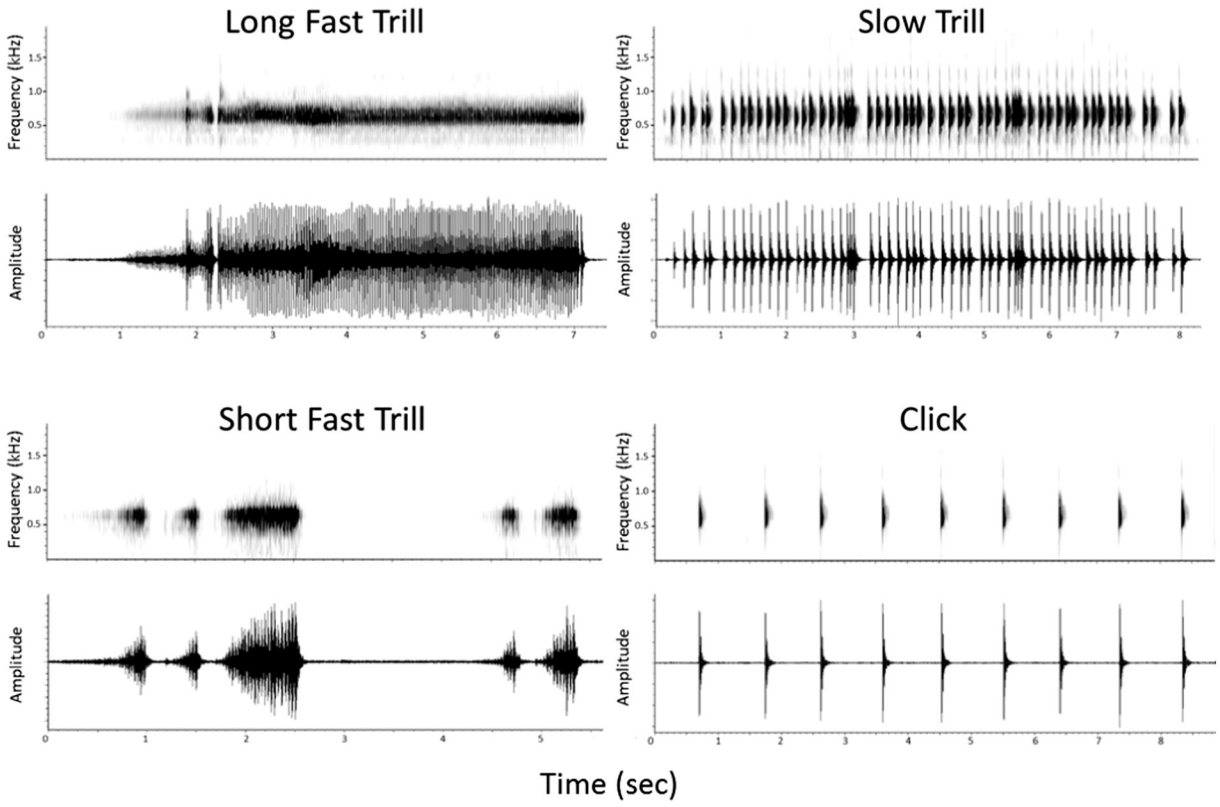

Fig. 1. *X. tropicalis* call types observed and described in this study: an individual long fast trill (upper left); a series of short fast trills (lower left); an individual slow trill (upper right); and a series of clicks (lower right). All calls have a dominant frequency of approximately 600 Hz. For each call type a spectrogram (frequency vs. time) is depicted in the top trace and a waveform (amplitude vs. time) is depicted in the bottom trace. See Table 1 for call type definitions.

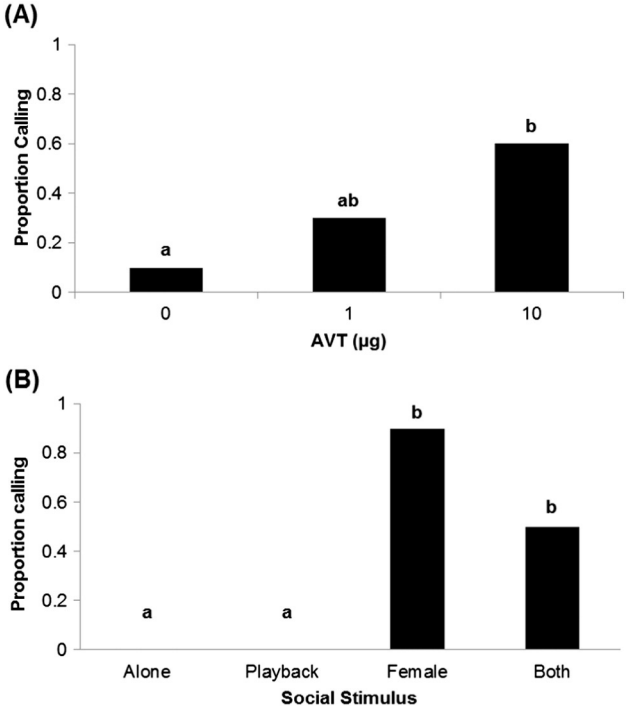

Fig. 2. Arginine vasotocin and social stimuli affect male calling behavior. (A) The proportion of males observed calling among AVT treatments in Experiment 1 was significantly different. Letters indicate significant differences from Fisher's exact test. (B) The proportion of calling males across all AVT treatments (10 total) in Experiment 1 was significantly different according to social context. Letters indicate significant differences from Fisher's exact test.

3.3. Experiment 2: effect of AVT and female stimulus on calling and clasping behavior

Discontinuous individual clicks were regularly recorded when males were alone and in the presence of a female; however, we could not conclusively rule out the possibility that females also produced clicks as a release call. Thus, frogs were considered to be calling only when producing trill-type calls. There were an equal proportion of frogs observed calling in all AVT treatment groups (Fig. 3A). For males that did call, there was no difference among treatment groups in the amount of time spent calling (Kruskal–Wallis, $P = 0.18$; Fig. 3B) or in the amount of time displaying the individual call types (Kruskal–Wallis, $P > 0.05$ for each call type; data not shown). However, the proportion of males that called according to social stimulus was significantly different among all calling frogs, regardless of AVT treatment ($G^2 = 24.59$, $P < 0.0001$; Fig. 3C). Specifically, more males called when females were available. There was a significant difference in the proportion of frogs that called when the male was alone compared to when the female was introduced (Fisher's exact, $P < 0.001$), when the female was introduced compared to when the female was removed (Fisher's exact, $P = 0.0045$) and when the female was removed compared to when the female was reintroduced (Fisher's exact, $P = 0.0045$). When males called, the duration (s) for each call type (mean ± SE, # callers) was 21.1 ± 12.2, 18 for long fast trills and 1.7 ± 0.7, 12 for short fast trills when paired with females; and 6.0 ± 1.9, 2 for long fast trills and 1.1 ± 0.2, 2 for short fast trills when alone. The proportion of males observed clasping did not differ according to AVT treatment ($G^2 = 2.96$, $P = 0.23$). One male was observed clasping when injected with 10 µg AVT, and two males were observed clasping when injected with 20 µg AVT. Males injected with the vehicle did not clasp.

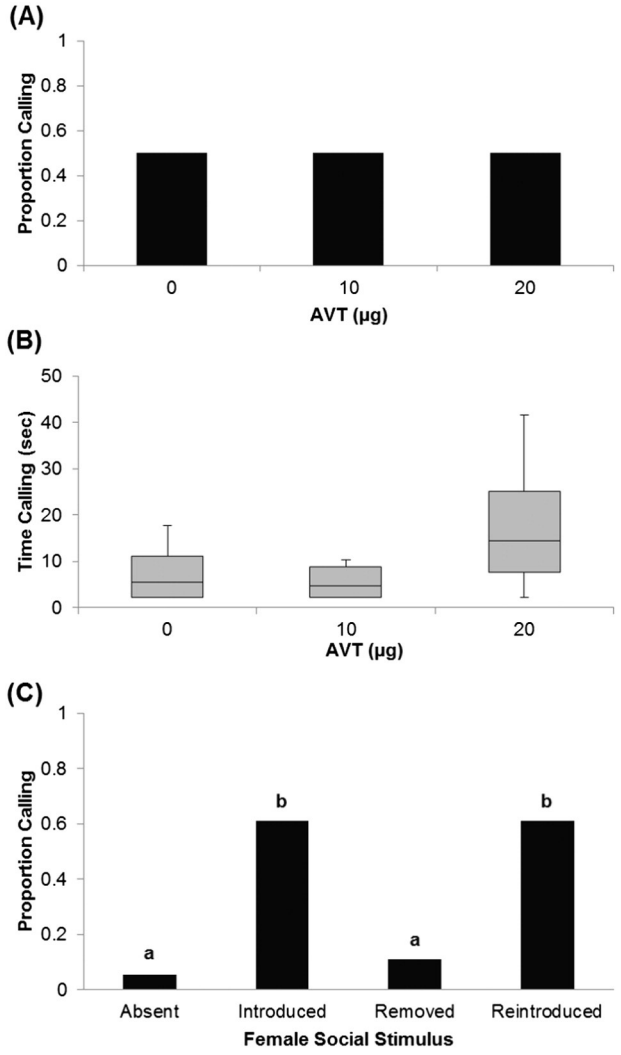

Fig. 3. Effect of arginine vasotocin and female stimulus on male calling behavior. (A) In Experiment 2 an equal proportion of males called in each AVT treatment. (B) The amount of time calling males called did not differ among AVT treatments. Box plot indicates median ± first and third quartiles while whiskers show minimum and maximum values. (C) Among calling males across AVT treatments in Experiment 2, the proportion of males that displayed calling behavior increased after a female was introduced. This proportion decreased when the female was removed then increased when the female was reintroduced. Letters indicate significant differences from post-hoc Fisher's exact test.

3.4. Experiment 3: effect of hCG on calling and clasping behavior

In Experiment 3 there was a significant difference among treatments in clasp attempts (Kruskal–Wallis, $P = 0.023$; Fig. 4A) and total time calling (Kruskal–Wallis, $P = 0.025$; Fig. 4B). Males receiving 10 IU hCG clasped significantly more than males receiving 5 IU hCG (Mann–Whitney U, $P = 0.028$). Males receiving 20 IU hCG clasped and called significantly more than males receiving vehicle and 5 IU hCG (Mann–Whitney U, $P < 0.01$ for each comparison). All other comparisons for total time calling and clasp attempts between treatments were not different. Additionally, there was a difference among treatments in time males displayed long fast trills (Kruskal–Wallis, $P < 0.01$; Fig. 4C) and slow trills (Kruskal–Wallis, $P < 0.01$; Fig. 4D). Males receiving 20 IU hCG displayed long fast trills significantly more compared to control and 5 IU treated males (Mann–Whitney U, $P < 0.01$). Males receiving 20 IU hCG displayed slow trills significantly more compared to control males (Mann–Whitney U, $P = 0.01$). Males receiving 5 IU hCG displayed slow trills for less time than male injected with 10 IU and 20 IU hCG (Mann–Whitney U, $P = 0.028$ and $P < 0.01$, respectively). All other comparisons between treatments for the display of call types did not differ.

3.5. Experiment 4: effect of AVT and hCG on calling and clasping behavior

There was a significant difference between treatments in clasp attempts (Mann–Whitney U, $P < 0.01$; Fig. 5A) and total time calling (Mann–Whitney U, $P = 0.012$; Fig. 5B) in Experiment 4 as males receiving hCG/AVT clasped and called significantly more than males receiving hCG only. Additionally, males receiving hCG/AVT displayed significantly more long fast trills compared to males treated with hCG alone (Mann–Whitney U, $P = 0.01$; Fig. 5C). Males receiving hCG/AVT also showed significantly more calling behavior (frogs calling > 100 s) than those treated with hCG only (Fisher's exact, $P < 0.01$; Fig. 5D). Comparisons between treatments for the display of all other call types were not significantly different (data not shown).

4. Discussion

Here, we show that AVT, as in other vocalizing vertebrates, has a stimulatory effect on calling behavior in male *X. tropicalis* when the appropriate social stimulus is present, specifically the presence of a female conspecific. Additionally, because we demonstrated that AVT increases both calling and clasping behavior in hCG-treated males, AVT may interact with gonadotropins and/or sex steroids to influence male reproductive behavior. Furthermore, our results demonstrate that the effect of AVT on sexual behavior in this species is context-dependent and suggest that visual, tactile, audio, and/or pheromonal cues from females may be important co-modulators of AVT's influence on sexual behavior.

In our study we also describe four call types displayed by *X. tropicalis* based on call duration and click rate characteristics of call bouts that we observed across all our experiments. Long fast trills were the most common calls observed in our experiments and are most similar to the advertisement calls identified in previous studies evaluating gonadotropin-induced calling in *X. tropicalis* [57,63]. Vigny [63] observed calls that were 1.5 s to 10 s in length with longer calls having a slower click rate, and Tobias et al. [57] described the call of *X. tropicalis* as having a single low frequency peak of approximately 500 Hz. In *X. laevis* male advertisement calls differ from female release calls (ticking) both in spectral frequency and click rate [55]. The fast trills, slow trills and clicks that we recorded in our study all had a single dominant frequency of approximately 600 Hz. Although rare, trills and clicks were also recorded in Experiment 2 when males were alone, and we observed single call bouts in which the click rate of a fast trill decreased to a slow trill or the click rate of a slow trill increased to a fast trill. Taken together, these results suggest that only the male vocalized when paired with a female; however, we excluded clicks from our analyses because male ticking cannot be reliably distinguished from female ticking in *X. laevis* [55]. While the fast trills that we observed are likely male advertisement calls, the behavioral responses elicited by the other call types described here are unclear and it is possible that the clicks we observed were actually produced by females. In fact, the clicks that we describe here are similar to clicks that are produced as a release call by unreceptive female *X. laevis* [55,58]. Further calling behavior studies and determining which sex produces which call may help us better understand vocal communication in this species.

In anurans, AVT treatment induces calling behavior in several frog species (see Introduction) but has not been previously shown to induce calling in any species from the family Pipidae. We provide evidence that AVT modulates vocal behavior in a new family of anuran amphibians. The effect of exogenous AVT on vocal behavior observed in our study is consistent with the results of other studies using other frog species as well as other vertebrate taxa. Vocal modulation by AVT and AVP is well-established in vertebrates [20] and has been demonstrated in

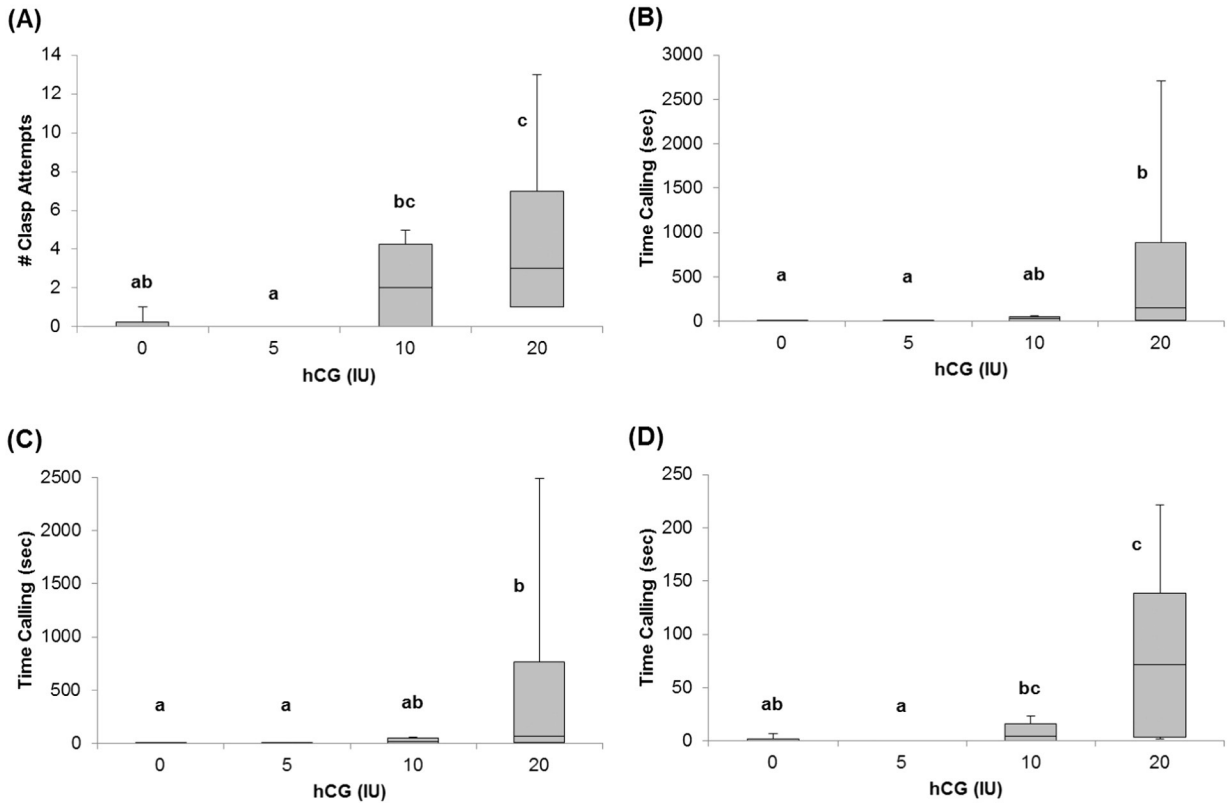

Fig. 4. Human chorionic gonadotropin affects male sexual behaviors. (A) There was a significant difference among treatment groups in the number of clasp attempts and (B) in the amount of time males called for Experiment 3. (C) There was a difference among treatment groups in the display of long fast trills and (D) in the display of slow trills among treatments. Details regarding box plots are described in Fig. 3B. Letters indicate significant differences from post-hoc analyses.

fishes [3,19], birds [17,32] and mammals [66,67]. For example, AVT administration induces vocalizations in the plainfin midshipman fish [19] and in white-crowned sparrows [32] while ultrasonic vocalizations are impaired in AVP 1b knockout mice [46]. AVP administration even affects social communication [53] and speech [62] in humans. Our results provide further evidence for the evolutionarily conserved role of the AVT/AVP neuropeptide system in modulating vocal behavior.

The absence of an AVT effect on calling behavior in Experiment 2 may have been caused by the natural endocrine state of all males used in this experiment. Androgen concentrations correlate with reproductive behaviors during the breeding season in many amphibian species. Testosterone (T) and/or dihydrotestosterone (DHT) levels are highest during the breeding season in several male anurans [23,24,36] and urodeles [12,51]. Additionally, plasma T levels correlate with calling behavior in anurans ([23,35,41,50,59]. O'Bryant and Wilczynski [41] also detected changes in both plasma T levels and brain AVT cell number in male green treefrogs during the breeding season. Furthermore, T and DHT treatment can restore reproductive behavior in castrated urodeles [2,11] and anurans [7] including calling in *X. laevis* [64]. Taken together, our results in Experiment 2 may be due to the animals already being primed with endogenous steroids prior to AVT injection.

hCG, a surrogate for luteinizing hormone, regulates testicular steroidogenesis in anurans [9,43]. Moreover, exogenous gonadotropin stimulates calling [55,63,64] and clasping [26,55] behaviors in *Xenopus*. Therefore, we evaluated the effect of hCG treatment on male reproductive behaviors in Experiment 3 to identify a dose of hCG that would induce a consistent baseline level of observable male sexual behavior in *X. tropicalis* for future AVT experiments. hCG induced a dose dependent elevation of clasping and calling behavior. Specifically, 20 IU hCG significantly elevated male sexual behavior compared to controls while the 10 IU dose did not differ from either treatment. Therefore, we determined that 10 IU hCG was adequate to prime male sexual behavior without over stimulating them and potentially concealing observable AVT effects on behavior. In Experiment 4, AVT co-treatment increased calling and clasping behavior compared to males treated with hCG only which provides further support for a role of AVT in reproductive behaviors in *X. tropicalis*. hCG increases circulating androgens, which are necessary for calling [64], and sex steroid treatment restores brain AVT concentrations and calling in castrated male anurans [6,42]. In addition, do-Rego et al. [14] reported that AVT stimulates neurosteroid synthesis in the frog brain. However, Yang et al. [70] recently reported that hCG treatment directly activates luteinizing hormone receptors in brain areas involved in call production in androgen-primed male *X. laevis*. Thus, our results suggest that AVT may be interacting with hCG directly or indirectly (via androgen synthesis) to impact *X. tropicalis* reproductive behavior. Use of this novel combined hormone treatment and its effect on sex steroid levels and the expression of neurohormone signaling systems may be important in subsequent behavior studies aimed to elucidate the mechanisms of complex signaling interactions between sex steroids and neuroendocrine systems for regulating social behaviors.

Social context is also important for inducing and influencing vocalizations in male anurans. In Experiments 1 and 2 male frogs, regardless of AVT treatment, displayed calling behavior significantly more when paired with a female than without a female suggesting female social cues are an important driver of male *X. tropicalis* vocalizations. Previous studies have used females as a stimulus when examining male vocal behavior in *Xenopus* species [26,57,63,64,70]. Although we cannot determine from this study if olfactory, visual, audio and/or tactile cues from females induce male behaviors, a pilot study in our lab suggests that male *X. tropicalis* prefer water that females had previously been housed

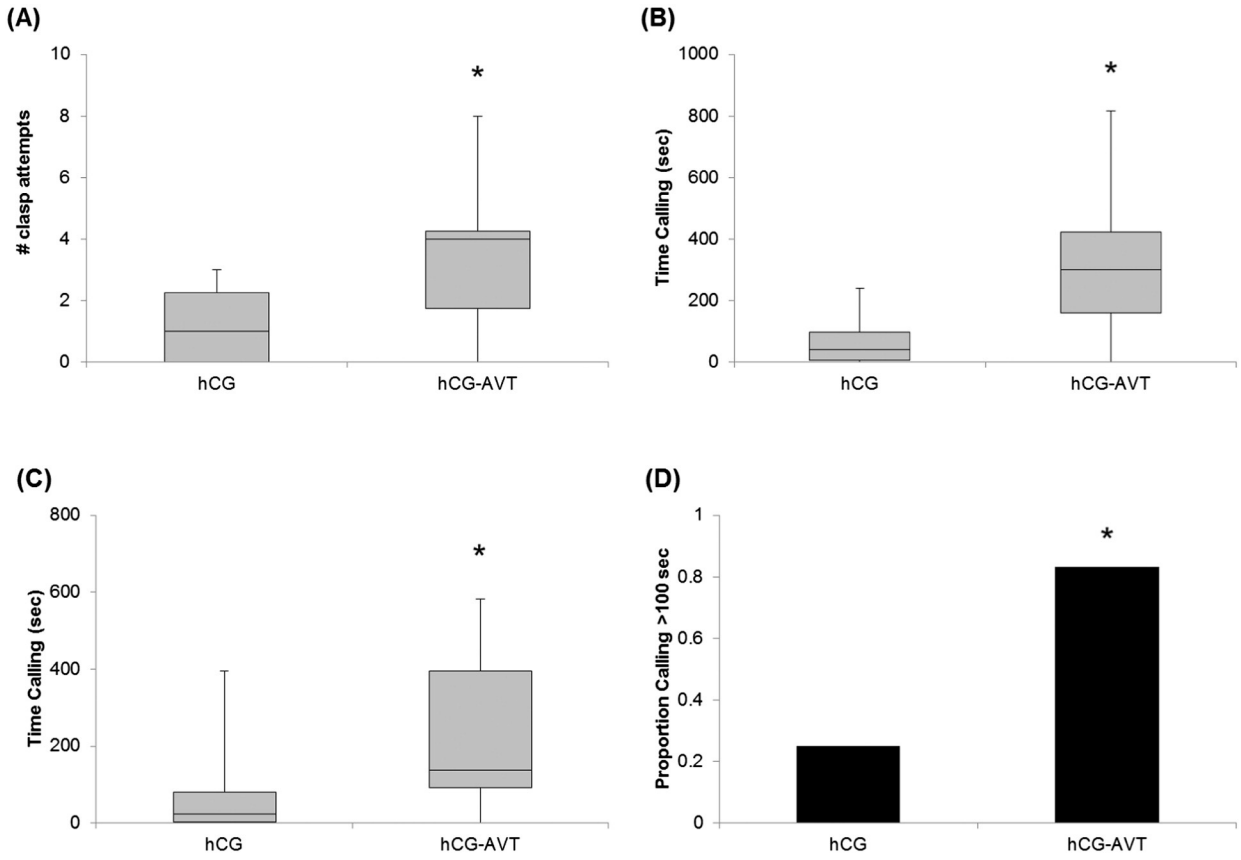

Fig. 5. Arginine vasotocin with human chorionic gonadotropin increases sexual behavior. (A) There was a significant difference between treatment groups in the number of clasp attempts and (B) in the amount of time males called for Experiment 4. (C) There was a difference between treatment groups in the display of long fast trills. (D) There was also a significant difference in the proportion of males calling >100 s. Details regarding box plots are described in Fig. 3B. Asterisks indicate a significant difference between treatments.

in compared to clean water (unpublished data), and in *X. laevis* calls from receptive females stimulate male clasping and calling behavior while calls from unreceptive females suppress these behaviors [58]. Thompson and Moore [54] also demonstrated that visual and pheromonal stimuli trigger courtship behavior in AVT-treated roughskin newts; however, AVT also can activate courtship behavior in male *Hynobius leechii* salamanders without exposure to any external stimuli [27].

Moreover, male behaviors in *X. tropicalis* could potentially be activated by stimuli from male conspecifics as Tobias et al. [55] found that hCG-treated male *X. laevis* call in the presence of other males and the amount of calling and types of calls produced are affected by the presence of the other male. In this study we did not evaluate calling behavior when males were paired together, but we used a call playback recording to simulate male–male interactions. Call playback experiments are commonly used to study anuran calling behavior [5,10,29], and Tobias et al. [56] found that playback of advertisement calls underwater affect calling in male *X. laevis*. Specifically, playback intensities that are equivalent to a nearby advertising male suppress vocal behavior while low-intensity playbacks are ineffective or can stimulate calling in the focal male [56]. We attempted to simulate a male conspecific using calls played over a loudspeaker; however, calling behavior was unaffected when males were exposed to a single low-intensity playback recording. Therefore, it remains unclear if the presence of a female or the presence of another frog, regardless of sex, affects calling behavior in male *X. tropicalis*. A future study evaluating the effect of specific sensory cues, male–male pairings or the alteration of call playback intensity would help clarify whether females or both sexes influence male *X. tropicalis* vocalizations.

Throughout the course of this study, males frequently called before and during clasping which has been previously documented in hCG-treated *X. laevis* [55]. AVT induces amplectic clasping in the roughskin newt [38], although it does not affect clasping in the Great Plains toad while GnRH affects this behavior in this species [44]. To our knowledge, we provide evidence for the first time that AVT induces clasping in an anuran and that it also affects both calling and clasping behaviors in the same species. Together, these results suggest that in different amphibian species similar behaviors are modulated by different neuropeptides.

In Experiment 4 AVT treatment increased the amount of time sexually active (hCG-treated) males produced long fast trills. These results suggest that AVT may influence call characteristics in *X. tropicalis*. In fact, AVT administration changed call characteristics that influence social interactions in gray treefrogs [30], altered mating calls in playback experiments with male túngura frogs [29], increased calling behavior during and after an agonistic encounter in male cricket frogs [10], and caused cricket frogs to produce calls characteristic of less aggressive males [34]. Together these results further suggest that AVT interacts with social cues to influence calling behavior in male anurans.

In conclusion, this study demonstrates that AVT induces reproductive behaviors in male *X. tropicalis*. To the best of our knowledge, this is the first evidence that AVT induces male calling and clasping behavior in a pipid anuran. AVT also interacts with hCG through signaling mechanisms still to be determined to influence these reproductive behaviors. Additionally, female social stimuli appear to be important for the expression of male reproductive behavior. Overall, our results suggest that *X. tropicalis* could be a useful neuroendocrine and neuroethological model to help further understand how hormone systems work to

modulate vocal and reproductive behaviors in amphibians and other vertebrates.

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