



Estradiol induces sexual behavior in female túngara frogs

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ABSTRACT

Steroid hormones play an important role in regulating vertebrate sexual behavior. In frogs and toads, injections of exogenous gonadotropins, which stimulate steroid hormone production, are often used to induce reproductive behavior, but steroid hormones alone are not always sufficient. To determine which hormonal conditions promote sexual behavior in female túngara frogs, we assessed the effect of hormone manipulation on the probability of phonotaxis behavior toward conspecific calls in post-reproductive females. We injected females with human chorionic gonadotropin (HCG), estradiol, estradiol plus progesterone, saline, or HCG plus fadrozole (an aromatase blocker) and tested their responses to mating calls. We found that injections of HCG, estradiol, and estradiol plus progesterone all increased phonotaxis behavior, whereas injections of saline or HCG plus fadrozole did not. Since injections of estradiol alone were effective at increasing phonotaxis behavior, we concluded that estradiol is sufficient for the expression of phonotaxis behavior. Next, to determine if estradiol-injected females display the same behavioral preferences as naturally breeding females, we compared mating call preferences of naturally breeding females to those of post-reproductive females injected with estradiol. We found that, when injected with estradiol, females show similar call preferences as naturally breeding females, although they were less likely to respond across multiple phonotaxis tests. Overall, our results suggest that estradiol is sufficient for the expression of sexual responses to mating calls in túngara frogs. To our knowledge, ours is the only study to find that estradiol alone is capable of promoting phonotaxis behavior in a frog.

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Introduction

Steroid hormones are important regulators of sexual behavior in vertebrates. In females, studies conducted on a variety of vertebrates have shown that estrogen plays an important role in facilitating sexual behavior (Ball and Balthazart, 2004; Moore et al., 2005). For example, both estrogen and progesterone are required for expression of estrous behavior and mating in rodents (Luttge et al., 1977). In reptiles, testosterone is known to facilitate female sexual behavior, which is in part due to aromatization of the hormone to estradiol (Noble and Greenberg, 1940; Winkler and Wade, 1998). In anurans (frogs and toads), however, there appears to be diversity in hormone–behavior relationships among species, with a variety of hormones implicated as being important.

In anurans, female sexual behavior can be expressed as movement towards conspecific calling males (phonotaxis) (Gerhardt and Huber, 2002), as producing vocalizations to attract males (Shen et al., 2008;

Tobias et al., 1998), or as the inhibition of behaviors typical of unreceptive females, such as release calls and leg extensions (Boyd, 1992; Diakow and Nemirow, 1981; Kelley, 1982). As in many other vertebrates, female anurans exhibit sexual behavior when they near oviposition (Lynch et al., 2005), a time when sex steroid hormones also tend to be high (Lynch and Wilczynski, 2005). A number of studies have found that injections of human chorionic gonadotropins (HCG) effectively increases sexual behavior in female frogs (Kelley, 1982; Lynch et al., 2006; Schmidt, 1984). HCG mimics the effects of endogenous gonadotropins and can stimulate the gonads to produce sex steroid hormones. Thus, these studies raise the possibility that, like other vertebrates, ovarian steroids regulate female sexual behavior in anurans. However, some studies suggest that sex steroids, alone, are insufficient to induce sexual behavior. For example, although receptivity to male clasping can be induced in ovariectomized *Xenopus laevis* with a combination of estradiol and progesterone, an additional injection of luteinizing hormone-releasing hormone caused females to be more sexually responsive compared to estradiol and progesterone injections alone (Kelley, 1982). Arginine vasotocin and/or prostaglandins are effective at inhibiting unreceptive calling behavior in the Northern leopard frog (Diakow and Nemirow, 1981) and *X. laevis* (Kelley, 1982; Weintraub et al., 1985). In the American toad, HCG induces phonotaxis, but its action is blocked by inhibition of prostaglandin synthesis (Schmidt, 1984). However,

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prostaglandin-induced phonotaxis appears to require progesterone (Schmidt, 1985a). In summary, it appears that there is significant diversity among anurans in the hormonal mechanisms underlying female sexual behavior.

Túngara frogs (*Physalaemus pustulosus*) have been a focus of sexual selection research. As a result, we know a great deal about their behavioral responses to mating calls (Ryan, 1985), and this makes them an excellent model for testing the effects of steroid hormones on female sexual behavior. Male túngara frogs produce a simple advertisement call that is a frequency-modulated “whine” (Rand and Ryan, 1981). Males can increase the attractiveness of the whine by adding up to 7 “chucks” to produce a complex “whine-chucks” call that is strongly preferred by females over the simple whine-only call (Rand and Ryan, 1981). Females express mating preferences by differential phonotaxis toward the call of choice, but females in this species do not produce advertisement calls.

Female túngara frogs go to ponds only on the night they are ready to mate (Ryan, 1985), and when unmated females are present at ponds, they have high concentrations of plasma estradiol and androgens (Lynch and Wilczynski, 2005). After a female chooses a mate and allows the male to clasp her in amplexus, she has high plasma estradiol and progesterone concentrations and low androgen levels (Lynch and Wilczynski, 2005). The high levels of estradiol and progesterone disappear within 7–10 days after the female has oviposited (Lynch and Wilczynski, 2005). In addition, injections of HCG, which increase plasma estradiol concentrations, raise the probability that a female will approach conspecific calls (Lynch et al., 2006). Together, these data suggest that estradiol and/or progesterone may be mediators of changes in female sexual behavior in this species. Therefore, we tested the effects of estradiol and progesterone on sexual motivation and female preferences for conspecific calls. Because HCG increases estradiol, as well as phonotaxis behavior, we first asked whether the HCG-induced increase in phonotaxis could be replicated by steroid hormone manipulation (Experiment 1). Our results suggest that estradiol is sufficient to increase phonotaxis. Therefore, we next asked whether estradiol injections elevate phonotaxis behavior to levels seen in naturally breeding females, and whether estradiol-injected females show the same call preferences as naturally breeding females (Experiment 2).

Experiment 1: which hormonal conditions promote phonotaxis behavior?

Methods

To determine which hormonal conditions promote phonotaxis behavior, we assessed the effects of hormone manipulation on the probability of phonotaxis behavior toward conspecific calls in post-reproductive females. To do so, we collected pairs during the breeding season, brought them back to the laboratory, and allowed them to make nests. Ten days after females had oviposited we injected all females with saline and tested them in phonotaxis behavior tests. Following the first set of phonotaxis tests, we injected females with one of five hormone treatments and tested them again with the same set of phonotaxis tests. Finally, to validate the hormone manipulations we bled the females to collect plasma to measure their hormone concentrations at the end of phonotaxis tests.

Frog collection

We collected adult females ($n=76$) individually or paired with males from breeding ponds between 19:00 and 23:00 h near Gamboa, Panamá in 2006. After capture, we placed amplexed pairs or individual females in plastic bags and brought them back to the Smithsonian Tropical Research Institute (STRI) laboratory. We paired

females that were caught individually with males that were calling in the same pond. We allowed the pairs to make foam nests after which we returned the foam nests and males to their original site of capture. We toe-clipped females for permanent identification following the recommended toe-clipping Guidelines for Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH) and the Society for the Study of Amphibians and Reptiles (SSAR). We measured the snout vent length (SVL) to the nearest 0.01 mm using digital slide calipers (Mitutoyo Corporation, Aurora, IL), and body mass to the nearest 0.1 g using a Pesola spring scale (Pesola, Baar, Switzerland). The mean SVL of females was 28.54 mm and the mean body mass at capture was 1.92 g. After oviposition, we kept the females at the STRI laboratory in Gamboa for 10 days before hormone manipulations because plasma hormone concentrations decline to non-breeding levels within 7–10 days after oviposition (Lynch and Wilczynski, 2005). During this time, we housed the females in 10-liter terrariums with substrate containing a mix of damp soil, leaf litter, and small twigs, and maintained them under ambient conditions (light: approximately 12 h 35 min from sunrise to sunset; temperature: approximately 28 °C). We provided the females with water, and fed them termites every other day. This work was approved by the University of North Carolina Institutional Animal Care and Use Committee (UNC IACUC) and was permitted by the National Authority for the Environment of Panamá (Autoridad Nacional del Ambiente).

Hormone manipulations

We followed one of two timelines for injections and phonotaxis testing for females in different treatment groups. Females from the HCG ($n=16$), estradiol (E; $n=16$), estradiol plus progesterone (E+P; $n=16$), and saline ($n=12$) groups were first injected with saline only followed 24 h later by phonotaxis testing. Females were then injected with either HCG (500 IU per g of body mass), E (0.07 µg per g of body mass), E+P (0.07 µg of E and 0.7 µg of P per g of body mass), or saline, and tested again 24 h later in the same phonotaxis tests. Females from the HCG plus fadrozole group (HCG+fad; $n=16$) followed the second timeline which was based on a previous study that demonstrated that fadrozole blocks HCG-induced estradiol production in túngara frogs (Lynch, 2005). We first injected females with saline followed by phonotaxis tests 24 h later. Females were then injected with a single dose of fadrozole (50 µg per frog), followed 24 h later by injections of fadrozole and HCG. Finally, another 24 h later we tested the females again in the phonotaxis tests. At the end of phonotaxis testing, all females were returned to their original site of capture. Each injection was 50-µl in volume and all substances were dissolved in saline (0.9% sodium chloride in water), although estradiol and progesterone were first dissolved in a small amount of ethanol. All substances were purchased from Sigma-Aldrich (St. Louis, MO) except fadrozole (4-(5, 6, 7, 8-tetrahydrimidazo [1, 5a] pyridine-5-yl) benzonitrile monohydrochloride), which was acquired from Novartis (Basel, Switzerland).

Phonotaxis tests

We conducted phonotaxis tests between 19:00 and 06:00 h. We tested each subject in four consecutive phonotaxis tests, each up to 15 min duration. In each test, the female heard two calls from opposing speakers. In the first and fourth tests we gave the females a choice between a conspecific whine (W) and a whine with 1 chuck (W1C) (see Stimuli, below). We separated tests 1 and 4 by up to 40 min during which we conducted two intervening tests to assess the ability of the females to choose between a conspecific and a heterospecific call, and between an artificial hybrid call and noise. We did not analyze the data from tests 2 and 3 due to low response from females. Instead, we used responses from tests 1 and 4 to determine a female's

willingness to approach conspecific calls. Specifically, females who approached either one of the conspecific calls in both tests were defined as showing “Persistent Phonotaxis.” Our definition of Persistent Phonotaxis is identical to the definition of receptivity used in prior studies (Lynch et al., 2005, 2006). We chose a different moniker so as to not confound our specific definition of behavior with the more general concept of sexual receptivity.

The phonotaxis chamber (1.5 m $W \times$ 1.5 m $L \times$ 1 m H) was made of mattress foam (Allegro Medical, Tempe, AZ) suspended by PVC pipes. We placed two audio speakers (Cambridge Soundworks, North Andover, MA) at equal distances from the center of the chamber. We set the peak intensity of the acoustic stimuli at 82 dB SPL measured from the center of the chamber where we released the female. We conducted the behavioral observations in a semi-dark room and from outside the chamber. We also ensured that the observer stayed still during testing to avoid any sudden movements that could have disturbed the female. The observer was not blind to the treatment groups. At the beginning of the phonotaxis tests we placed each subject in the center of the chamber under an inverted funnel for 3 min. During these 3 min, acoustic stimuli were broadcast antiphonally from the two opposite speakers with a 1 s delay between presentations. To control for side bias, we alternated the side on which each stimulus was presented in tests 1 and 4 for each individual female. We lifted the funnel 3 min after the start of the broadcasts and allowed the female up to 15 min to respond during which time the stimuli continued to be broadcast. Females had to approach within 10 cm of a speaker to have made a choice. We regarded the female as non-responsive if she remained stationary for more than 5 min after the funnel was lifted, or if she did not approach within 10 cm of a speaker. For the females that showed Persistent Phonotaxis, we calculated the mean latency to respond (time to approach within 10 cm of a speaker after the funnel was lifted) in the first and fourth phonotaxis tests.

Stimuli

We used natural túngara calls recorded from the Gambia population, and we assembled all stimuli on a Macintosh computer using Raven Version 1.2.1 (Cornell Laboratory of Ornithology, BioAcoustics Research Program) and Garageband (Apple, Cupertino, CA). To maximize the generalizability of our conclusions (Kroodsma, 1989; Wiley, 2003), we used multiple call exemplars as follows. We used 7 pairs of mating calls recorded from 7 different males. Each pair of mating calls consisted of a W and a W1C call from the same male. In each phonotaxis test, the female was presented with a pair of calls recorded from an individual male. No individual female heard the same pair of calls twice during the course of the experiment. All stimuli were adjusted to the same peak amplitude.

Hormone assays

To validate the endocrine manipulations, total estradiol and progesterone concentrations were determined using enzyme immunoassay kits (Cayman Chemicals, Ann Arbor, Michigan). To collect plasma, we bled frogs from the retro-orbital sinus using a heparinized microcapillary tube, centrifuged the blood samples at 6000 rpm for 4 min, and stored the plasma supernatant at -20°C until later analysis. Plasma volumes ranged from 5–40 μl for individual frogs. If we had less than 20 μl of plasma, we could not conduct both hormone assays on the same sample. Therefore, sample sizes vary. Plasma samples were extracted twice with 2 ml of ether, evaporated, and then resuspended in enzyme immunoassay buffer. Recovery estimations were performed according to the Cayman kit instructions. These kits had previously been validated in this species (Lynch and Wilczynski, 2005; Lynch et al., 2006). However, we also validated the kits by adding known and unknown amounts of hormones to samples and measuring them

repeatedly in different assays. The mean recovery after extraction was 52% for estrogen and 56% for progesterone, respectively. Recovery values were used to correct the concentration of hormone estimated in each sample. Each sample was assayed at two dilutions and each dilution was assayed in duplicates. The dilution value that fell within the most sensitive part of the standard curve generated from each assay was subsequently used for calculation of plasma samples. In total, five separate estradiol and five progesterone assays were conducted to analyze all the samples. Inter-assay variation was 18.4% and 9.65% for estrogen and progesterone, respectively. Cross reactivity in the estrogen kit was 0.1% for testosterone and 5- α -DHT, 0.07% for 17 α -estradiol, and 0.03% for progesterone with a detection limit of 8 pg/ml. Cross reactivity in the progesterone kit was 7.2% and 0.01% for 17 β -estradiol and 17 α -estradiol respectively, with a detection limit of 10 pg/ml. Samples that were measured at the lowest dilution but were outside the sensitive area of the standard curve (i.e. very low amounts of hormone present in the plasma) were assigned the lowest detectable amount for the assay.

Statistical analyses

We analyzed plasma estradiol and progesterone concentrations for all treatment groups using a one-way ANOVA and we conducted least significant difference (LSD) post-hoc analyses to examine pair-wise differences in hormone concentrations among the treatment groups. We used McNemar's 'test of significant change' (Zar, 1999) to determine whether, for each group, hormone treatment changed the probability of showing Persistent Phonotaxis compared to the initial saline injection. McNemar's test takes into account the within-subject nature of this comparison. We used Fisher's exact chi square to compare the effects of hormone treatment on the probability of Persistent Phonotaxis directly to one another. In addition, among females that showed Persistent Phonotaxis in the E, E+P, and HCG groups, we used ANOVA to test for the effect of hormone treatment on the latency to respond to calls. We did not include females from the saline or HCG+fad groups in this analysis since the number of females that showed Persistent Phonotaxis in these groups was 2 and 3, respectively. Throughout, instead of using a threshold alpha level to interpret our results, we describe the pattern of results and use p values to support our statements as recommended by Hurlbert and Lombardi (2003) and Stewart-Oaten (1995). We consider p to be a continuous variable and we consider lower p values to represent a lower probability of incorrectly rejecting the null hypothesis of no difference.

Results

Estradiol injections successfully elevated plasma estradiol concentrations and generated substantial variation in estradiol concentrations among groups ($F_{4,45}=5.872$, $p<0.001$; Fig. 1). Estradiol injections increased estradiol concentrations by approximately three-fold compared to saline-treated females. The magnitude of the change in estradiol concentrations is comparable to that observed in amplexed females compared to post-reproductive females (Lynch and Wilczynski, 2005), although absolute levels of estradiol of all groups were lower in that earlier study. Unlike previous studies (Lynch et al., 2006), HCG injections did not increase estradiol concentrations significantly above females injected with saline or HCG+fad. In contrast to estradiol levels, we did not detect a substantial difference in progesterone concentrations among the treatment groups ($F_{4,47}=1.60$, $p=0.190$; Fig. 1). Although we were surprised that our hormone manipulation did not elevate progesterone concentrations, a prior study similarly failed to elevate progesterone concentrations using HCG in túngara frogs (Lynch and Wilczynski, 2008).

Females injected with estradiol showed the highest rates of Persistent Phonotaxis (75%), followed by those injected with E+P

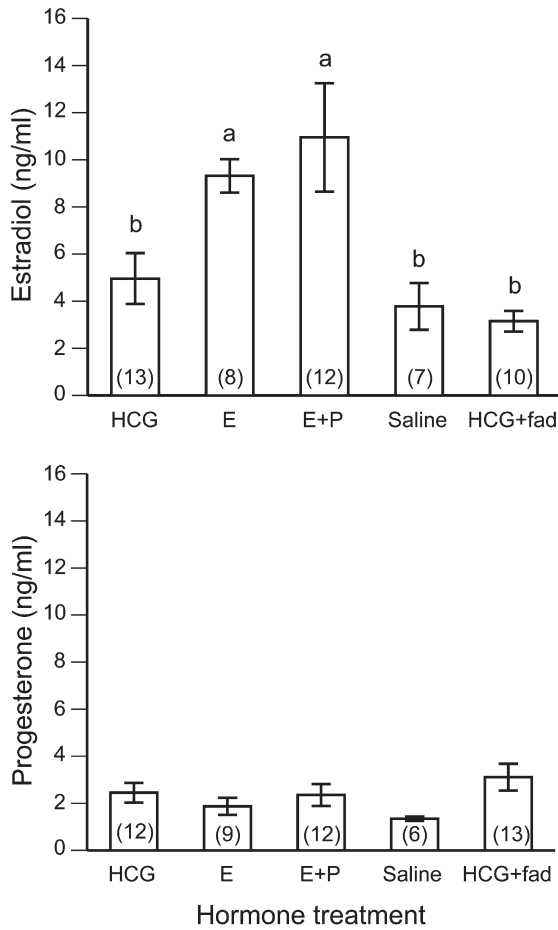


Fig. 1. Plasma estradiol and progesterone concentrations (mean ± SE) 24 h after the final injections in Experiment 1. Final injections were human chorionic gonadotropins (HCG), estradiol (E), estradiol plus progesterone (E+P), saline, or a combination of HCG and the aromatase inhibitor fadrozole (HCG+fad). Sample sizes are shown in parentheses and common letters indicate groups that are statistically indistinguishable at $p < 0.05$.

(56%) and HCG (44%). Compared to when the same females were injected with saline, the increase in Persistent Phonotaxis was strong for estradiol ($\chi^2 = 11.0, p < 0.001$) and HCG ($\chi^2 = 6.0, p = 0.014$), but was more modest for E+P ($\chi^2 = 2.8, p = 0.096$) because of higher rates of Persistent Phonotaxis after initial saline injections in this group (Fig. 2). In contrast, females injected with HCG+fad did not change their probability of Persistent Phonotaxis compared to when they were injected with saline ($\chi^2 = 0.0, p = 1.0$; Fig. 2), nor did females who received a second injection of saline ($\chi^2 = 1.0, p = 0.32$; Fig. 2). These results suggest that injections of HCG, E, and E+P all increase the probability of phonotaxis. In order to determine if the hormone injections increased Persistent Phonotaxis to different levels, we compared the effect of hormone treatments directly to one another. We found that females injected with E alone had similar rates of Persistent Phonotaxis as females injected with E+P ($\chi^2 = 1.3, p = 0.46$). Compared to HCG-injected females, both E-injected females ($\chi^2 = 3.3, p = 0.15$), and E+P-injected females ($\chi^2 = 0.5, p = 0.72$) showed similar rates of Persistent Phonotaxis. Furthermore, among females showing Persistent Phonotaxis in the E, E+P, and HCG-treated females, hormone treatment had no effect on latency to respond to conspecific calls ($F_{3,27} = 0.256, p = 0.86$). The mean ± SE latency to respond in the E, E+P, and HCG-treated females were 333.83 ± 40.01 , 358.2 ± 42.9 , and 321.5 ± 76.1 s, respectively. Thus, injections of HCG, E, and E+P had similar effects on the motivation to approach conspecific calls.

Experiment 2: does estradiol elicit natural responses to mating calls?

Methods

Results of Experiment 1 show that estradiol was sufficient to increase phonotaxis to levels observed in HCG-injected females. However, that experiment did not test whether estradiol-injected females show a similar degree of sexual motivation as observed in naturally breeding females, or whether they display the same call preferences as naturally breeding females. Therefore, we next compared phonotaxis responses of females tested right after capture to when they were post-reproductive and injected with either estradiol or saline. In this experiment, we assessed sexual motivation as the probability of Persistent Phonotaxis and as the probability of approaching a speaker during any given test. As a reminder, females who approached either one of the conspecific calls in the first and last tests were defined as showing Persistent Phonotaxis.

Frog collection and hormone manipulation

Experimental procedures were identical to Experiment 1, except where noted. In 2007, we collected 48 amplexed females from breeding ponds between 20:00 and 24:00 h near Rio Piro on the Osa Peninsula in Costa Rica. The mean SVL of females was 29.63 mm and the mean body mass at capture was 1.84 g. After capture, we removed the male and tested the female's behavior in a series of two-choice phonotaxis tests within 10 h of capture at the Osa Biodiversity Research Station. We then returned the females to their mate to allow the pairs to complete nesting, and we housed females in terrariums under ambient conditions (approximately 12 h 20 min from sunrise to sunset and 28 °C). Ten days following oviposition, we injected females with estradiol ($n = 33$), or saline ($n = 15$), and 24 h after injection tested their behavior in the same series of two-choice tests. This work was approved by the UNC IACUC and permitted by Costa Rica's Ministerio del Ambiente Y Energia (MINAE) and Sistema Nacional de Áreas de Conservación (SINAC).

Phonotaxis tests

We tested each subject in five consecutive phonotaxis choice tests between 19:00 and 05:00 h. Basic phonotaxis procedures were

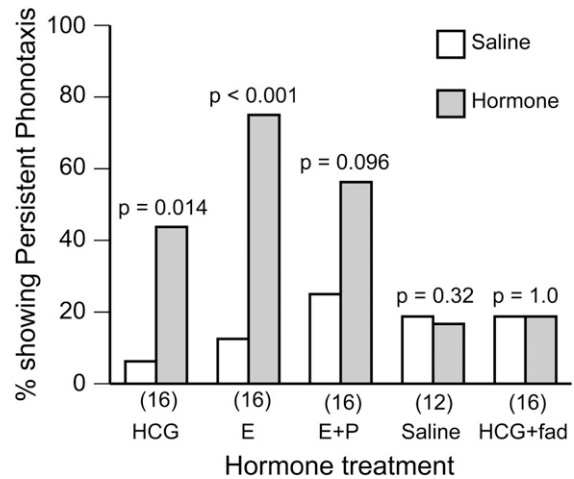


Fig. 2. Effects of hormonal manipulation on the probability of showing Persistent Phonotaxis to conspecific mating calls in Experiment 1. Females were categorized as showing Persistent Phonotaxis if they approached one of two conspecific mating calls in two different phonotaxis tests. Persistent Phonotaxis was first assessed after injection with saline and then after 1 of 5 different hormone injections. Hormone treatments were human chorionic gonadotropins (HCG), estradiol (E), estradiol plus progesterone (E+P), saline, or a combination of HCG and the aromatase inhibitor fadrozole (HCG+fad).

identical to those in Experiment 1, except we used Tivoli Portable Audio Laboratory speakers (Tivoli Audio, Cambridge MA). In order to test a range of responses, we included phonotaxis tests where clear and strong preferences have been well documented, as well as tests for which we expected no strong call preferences, as follows. Tests 1 and 5 assessed the preference for the complex whine–chuck call over the simple whine, and test 2 assessed the preference for a conspecific whine–chuck call over a heterospecific whine. In these cases, a strong preference for the whine–chuck call over the alternative is well documented (Griddi-Papp et al., 2006; Ryan, 1980). Tests 3 and 4 compared responses to conspecific whine–chuck calls that varied in the number of chucks. In tests 3 and 4, females were assessed for their preference for a whine with 1 chuck over a whine with three chucks, and a whine with six chucks, respectively. Prior studies have shown that, at the amplitudes used in our experiment, females do not discriminate among whine–chuck calls based on the number of chucks (M. J. Ryan, personal communication).

Stimuli

We used natural túngara calls recorded near Puerto Jiménez on the Osa Peninsula; the heterospecific whines were recorded from *Physalaemus enesefae (fischeri)* in Venezuela. We assembled all stimuli on a Macintosh computer using the software programs Raven and Audacity (audacity.sourceforge.net). We used call exemplars from 4 different male túngaras and 4 different *P. enesefae* males. For the túngara calls, the W and W1C calls were unmanipulated calls. To create the calls with multiple chucks, we added two (W3C) or five (W6C) chucks to the end of the W1C calls with 50 ms of intervening silence. For the túngara call stimuli, we presented each female with stimuli from the same male, and females were presented with the same set of stimuli when they were tested under both hormonal conditions. Call exemplars were distributed among the different treatment groups.

Statistical analyses

We assessed sexual motivation as the probability of Persistent Phonotaxis (approaching either conspecific call in the first and last tests), and the probability of responding during a test (approaching any speaker during a given test). As in Experiment 1, we used McNemar's test of significant change to assess the effect of hormonal condition on the probability of showing Persistent Phonotaxis. We used Fisher's exact chi square to compare the probability of responding in each phonotaxis test when tested after amplexus versus after estradiol injection. Because chi square assumes independence of each observation, we assigned each female to one of two groups as follows. To represent amplexed females ($n=15$), we included the responses generated following amplexus of females in the saline group. To represent the estradiol group, we included the responses generated following estradiol injection of the estradiol-treated group ($n=33$). Thus, each female was only included once in these analyses.

Finally, we used Fisher's exact chi square to assess the effect of estradiol injection on call preferences in comparison to amplexus. To do so, for each phonotaxis test, it was necessary to only consider a female's response once in order to satisfy the assumption of independence. Our strategy for sorting the data was designed to maximize the sample sizes representing each group. Females were included in the amplexed group if they were originally assigned to the saline treatment group or if they were originally assigned to the estradiol treatment group but failed to respond after estradiol injection. Females were included in the estradiol treatment group if they were injected with estradiol and responded. Since we considered each preference test separately, sample sizes varied for each analysis.

In order to facilitate direct comparisons between amplexed females and estradiol-injected females, we expressed the data as the number of females who chose the W1C, since this call was common to all phonotaxis tests. We conducted analyses of preferences for the W1C call in test 1 (W1C vs. W), test 2 (W1C vs. Het), and test 3 (W1C vs. W3C). We excluded analyses of test 4 because the number of estradiol-injected females that responded during that test was prohibitively low ($n=8$). We did not include an analysis of test 5 because it was redundant with test 1.

Results

Overall, a high percentage of amplexed females showed Persistent Phonotaxis. Compared to when they were amplexed, saline-injected females were less likely to show Persistent Phonotaxis ($\chi^2=6.0$, $p=0.014$; Fig. 3). Females that were injected with E had similar probability of Persistent Phonotaxis compared to when they were tested after amplexus ($\chi^2=1.6$, $p=0.21$; Fig. 3), suggesting that E-injected females exhibit similar levels of motivation to respond to calls as naturally breeding females. In addition, we found that amplexed females were more responsive across tests compared to E-injected females (Fig. 4A). Specifically, E-injected females were less likely to respond in tests 2–5 compared to amplexed females (test 1: $\chi^2=0.18$, $p=1.0$; test 2: $\chi^2=6.4$, $p=0.037$; test 3: $\chi^2=4.1$, $p=0.065$; test 4: $\chi^2=7.9$, $p=0.009$; test 5: $\chi^2=7.7$, $p=0.041$). Qualitatively, saline-injected females showed a similar decline in responses during tests 2–5 (data not shown), but we could not test this statistically due to low sample sizes. Nonetheless, the similar response of saline- and E-injected females suggests that this decline in responsiveness is not a result of estradiol treatment, *per se*, but is more likely due to some aspect of housing, passage of time, or being injected.

We also compared call preference of amplexed females to E-treated females. We found that amplexed females chose the W1C about 83% of the time and E-injected females chose the W1C 74% of the time (test 1: $\chi^2=0.55$, $p=0.72$; Fig. 4B), demonstrating that the preference for the complex whine–chuck call is intact in E-injected females. In test 2, all females showed a strong preference for the conspecific W1C call over the heterospecific whine regardless of reproductive condition ($\chi^2=3.5$, $p=0.18$; Fig. 4B). In addition, we found that females chose the W1C over W3C about 63% of the time,

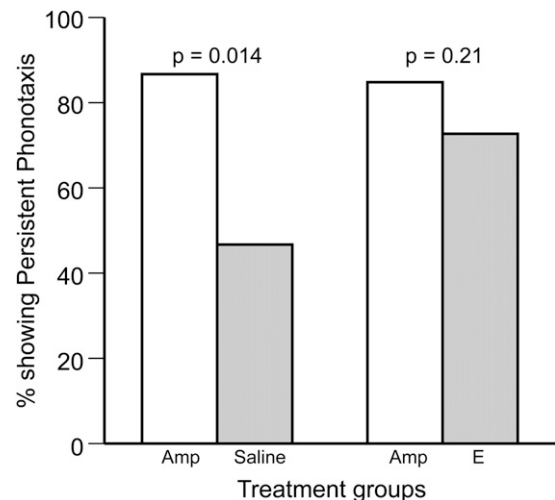


Fig. 3. Effects of hormonal condition on the probability of showing Persistent Phonotaxis to conspecific mating calls in Experiment 2. Females were categorized as showing Persistent Phonotaxis if they approached one of two conspecific mating calls in two different phonotaxis tests. Persistent Phonotaxis was first assessed within 10 h of amplexus (Amp), and then 11 days later after an injection of saline ($n=15$), or estradiol (E; $n=33$).

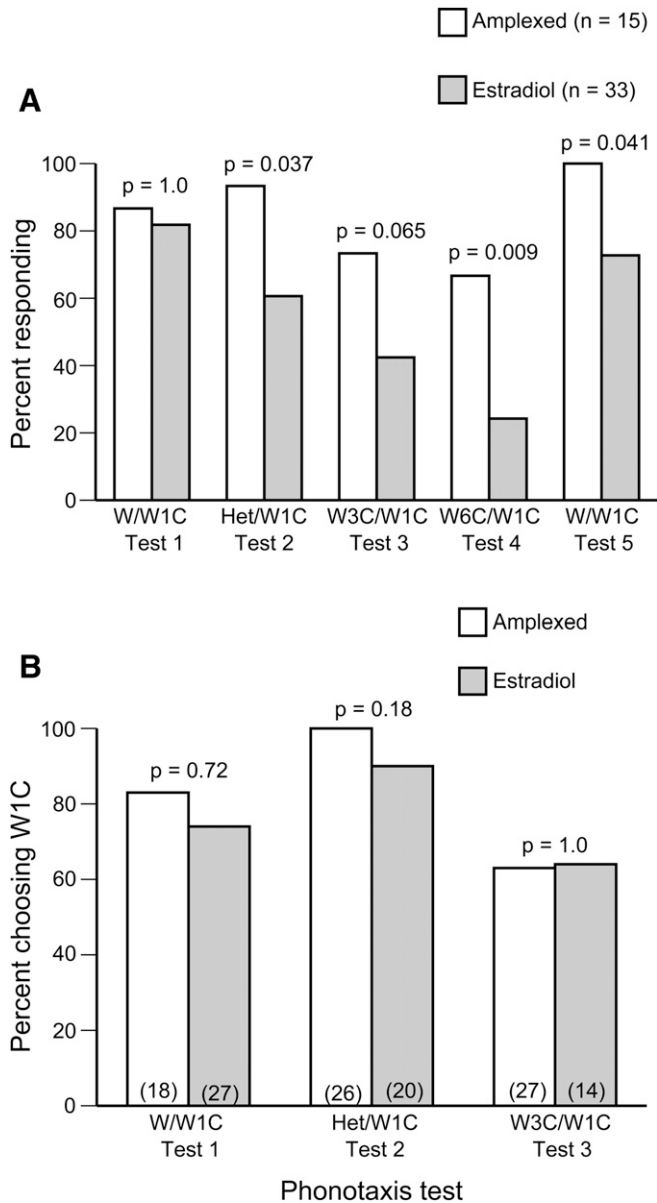


Fig. 4. (A) Effects of hormonal condition on the probability of responding during 5 sequential phonotaxis tests in Experiment 2. Females were considered responsive if they approached any speaker during a test. Females were first tested within 10 h of amplexus ($n = 15$), and then 11 days later after an injection of estradiol ($n = 33$). (B) Effect of hormonal condition on the preference for the whine+1 chuck call (W1C) in 3 sequential phonotaxis tests in Experiment 2. Females were either tested within 10 h of amplexus or 11 days later after injection with estradiol. Sample sizes (indicated in parenthesis) vary depending on the proportion of females that responded in each test.

regardless of whether they were tested after amplexus or after E injection ($\chi^2 = 0.007$, $p = 1.0$; Fig. 4B). In summary, E-injected females show similar call preferences as amplexed females.

Discussion

We found that injections of human chorionic gonadotropins (HCG), estradiol (E), and estradiol plus progesterone (E+P) all increased phonotaxis behavior, whereas injections of saline or HCG plus fadrozole (HCG+fad) did not. Since injections of estradiol alone were effective at increasing phonotaxis behavior, we conclude that estradiol is sufficient for the expression of phonotaxis behavior, a critical feature of sexual behavior in female túngara frogs. We also found that estradiol-injected females were just as likely to show

phonotaxis, and expressed similar call preferences, as females in natural breeding condition. Prior evidence from HCG manipulations and hormonal studies of naturally breeding females have shown that the expression of sexual behavior in female túngara frogs is accompanied by elevated estrogen and progesterone concentrations (Lynch and Wilczynski, 2005; Lynch et al., 2005, 2006). Taken together, these data suggest that the natural changes in female sexual behavior that occurs over the reproductive cycle is controlled primarily by fluctuations in estradiol concentrations.

Our hormonal manipulations show that injections of estradiol alone can increase Persistent Phonotaxis (approaching either conspecific call in both the first and last phonotaxis tests) leading to our conclusion that estradiol is sufficient for sexual responses to mating calls. However, whether estradiol is necessary for phonotaxis remains unclear. Although we found that HCG injections effectively increased phonotaxis behavior, they failed to substantially elevate estradiol concentrations, suggesting that HCG could modulate phonotaxis behavior in an estradiol-independent manner. Nonetheless, combining HCG with the aromatase inhibitor fadrozole blocked HCG-induced phonotaxis. Although estradiol levels in the HCG+fad group were similar to saline-injected females, we were unable to conclude that fadrozole blocked HCG-induced phonotaxis by inhibiting estradiol since HCG alone failed to substantially elevate estradiol. Thus, it is possible that fadrozole inhibited phonotaxis through some estradiol-independent pathway. Since an earlier study using the same injection protocol demonstrated that fadrozole blocks HCG-induced production of estradiol in túngara frogs (Lynch, 2005) we suspect that the ambiguity in our data stems from our inability to demonstrate elevated levels of estradiol in our HCG-injected females. Regardless, future studies will be necessary to determine whether estradiol is necessary for phonotaxis behavior in female túngara frogs. In addition, we cannot draw strong conclusions about the role of progesterone from our data, since we were unable to demonstrate that our injections increased progesterone concentrations to breeding levels (~ 20 ng/ml; Lynch and Wilczynski, 2005). It is possible that the progesterone dose that we used was not sufficiently high, that we failed to detect an increase in progesterone with the timing of our sampling, or that our progesterone assay failed. Nonetheless, since we did not observe any significant difference in the expression of sexual behavior among E-, E+P-, and the HCG-injected females, it appears that progesterone is not necessary for phonotaxis. However, it remains to be conclusively determined whether progesterone modulates sexual behavior in the túngara frog.

Estradiol-injected females were similar to amplexed females in the probability of showing Persistent Phonotaxis and in their call preferences. Females injected with estradiol displayed strong preferences for the complex whine-chuck call over the simple whine, and for a conspecific call over a heterospecific call. They also failed to discriminate among calls based on the number of chucks in a manner similar to amplexed females. These data suggest that estradiol-induced phonotaxis behavior is indistinguishable from that of amplexed females. However, estradiol-injected females showed a decline in the probability of responding across sequential phonotaxis tests. Because saline-injected females seemed to show a similar decline, the waning of phonotaxis responses may be a consequence of housing or injection, and not a consequence of estradiol treatment *per se*. Nonetheless, estradiol-injected females were less reliable in their phonotaxis behavior than amplexed females, suggesting that, under these conditions, estradiol was unable to induce sexual motivation to levels as seen in amplexed females tested on the night of capture. Thus, estradiol injections are highly effective at inducing sexual behavior that is similar to naturally breeding females, but some differences in sexual motivation appear to exist.

Prior work suggests diversity in hormone-behavior relationships among anurans, although studies of different species do not always manipulate the same combination of hormones, making direct

comparisons difficult. HCG has commonly been used to induce sexual behavior in frogs, including female phonotaxis (Lynch et al., 2006; Schmidt, 1984). Presumably, HCG acts by mimicking endogenous gonadotropins to stimulate the production of ovarian hormones. HCG could also directly bind to luteinizing hormone receptors to affect behavior (Yang et al., 2007). To our knowledge, ours is the only study to demonstrate that estradiol alone is effective at inducing phonotaxis behavior in an anuran. In *X. laevis*, steroid hormones are effective at promoting receptivity to amplexus, but a combination of estradiol and progesterone is necessary (Kelley, 1982). In addition, in the American toad, HCG-induced phonotaxis depends on the production of prostaglandins (Schmidt, 1984) but prostaglandin-induced phonotaxis may require progesterone (Schmidt, 1985b); the priming effects of estradiol alone were not tested. It is worth noting, however, that the primary goal of these prior studies was to develop a pharmacological method for inducing phonotaxis, and they were not designed to discover the natural hormonal mechanisms of phonotaxis (Schmidt, 1984; Schmidt, 1985a; Schmidt, 1985b). Nonetheless, the effects of prostaglandins on phonotaxis appear to be potent (Schmidt, 1985b).

Prostaglandins are non-steroid fatty acid hormones produced in many tissues, including the ovaries, and are associated with ovulation, oviposition, parturition, and sexual receptivity in widespread taxa (Gobbetti and Zerani, 1992; Gobbetti and Zerani, 1999; Guillette et al., 1991). Several studies have demonstrated reciprocal relationships between estradiol and prostaglandins, including the stimulation of aromatase activity by prostaglandins (Gobbetti and Zerani, 1992) and the stimulation of prostaglandin synthase expression by estradiol (Wu et al., 2005). Thus, it is possible that our estradiol manipulations were effective at inducing phonotaxis in túngara frogs, in part, through stimulation of prostaglandin production, or that prostaglandin injections in prior studies were effective because they also increased estradiol concentrations. If so, it would suggest that our results are not inconsistent with prior studies. Future studies of the interactions between steroid hormones and prostaglandins are necessary for a more complete understanding of the hormonal mechanisms of female sexual behavior in anurans.

Theoretical models suggest that both intrinsic and extrinsic factors may serve as constraints that can influence mate choice decisions (Jennions and Petrie, 1997). Intrinsic factors, such as hormonal state, can modulate female sexual behavior by allowing the female to be plastic in her mate choice behavior (Lynch et al., 2005). Our study demonstrates that estradiol can induce sexual behavior in female túngara frogs, which suggests that steroid hormones are capable of inducing female mate choice behavior via modulation of neural pathways. Clearly more studies are needed to investigate the precise neuroendocrine mechanisms by which estradiol modulates sexual motivation and mate choice behavior in the túngara frog. Because female frogs base mate choice decisions largely on acoustic signals produced by males, anurans are an attractive model for investigating the effect of steroid hormones on the neural pathways that modulate sexual behavior. Our results illustrate an important proximate mechanism that could have an essential function in influencing female mate choice behavior in anurans within the context of sexual selection.

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