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 Nemiroff, 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 ovariecto-mized *Xenopus laevis* with a combination of estradiol and progester-one, 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 Nemiroff, 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,

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