BEHAVIORAL ENDOCRINOLOGY OF FEMALE GRAY TREEFROGS, *HYLA VERSICOLOR*, IN RESPONSE TO ACOUSTIC STIMULATION.

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Doctor of Philosophy

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

Coordination of reproduction is a fundamental problem for the sexes in most animals. For most males the solution is generally to maintain a relatively constant state of readiness with a short refractory period. This is not usually an option for females, given the larger material investment typically required for female gamete production. Particularly in organisms with a limited breeding period, females must rely on both external and physiological cues to regulate the phenology of their reproduction and behavior.

I investigated how the social environment might influence the physiology and behavior of female reproduction. Male signals, used for mate attraction and male-male competition, are a prominent feature of the breeding environment in many species. I studied the relationship between male acoustic signals, and the endocrinology and behavior of females in the gray treefrog, *Hyla versicolor*. Gray treefrog males form dense choruses at breeding ponds during a breeding season, a period of several months. These choruses are audible up to several hundred meters away, where females spend most of the year. In this species of treefrog, females appear to evaluate males solely on call characteristics, making it ideal for studies investigating acoustic influences. It is not uncommon for female gray treefrogs to produce multiple clutches in a single season.

Hormonal levels fluctuate during the breeding season in many anurans, but the identity of the hormones that modulate breeding behavior and their effects remain unclear. We tested the influence of a combined treatment of progesterone and prostaglandin on phonotaxis, the key proceptive reproductive behavior of female anurans. Injections of progesterone and prostaglandin elevated estradiol levels and promoted phonotaxis in a manner similar to naturally breeding females, suggesting these hormones may influence or regulate this reproductive behavior.

Using field sampling of wild frogs I determined that females show the greatest elevation of steroid hormones on breeding nights, and that non-breeding females have elevated levels of estradiol and testosterone during the breeding season relative to the non-breeding season. This was important to demonstrate that reproductive events and hormonal elevation coincide, and that male acoustic signals and elevation of hormones in females are correlated events. I then conducted several experiments to determine the role acoustic signals might be playing in these observed changes in steroid levels.

Over the time scale of an entire breeding season, I exposed captive treefrogs to different acoustic treatments and found that females that heard conspecific signals were not more likely to elevate the steroids associated with reproductive events than were females that heard control stimuli. Females that heard calls on previous nights were also not more likely to seek out or amplex calling males than females that heard control stimuli. Thus I determined there was no evidence that male acoustic signals influenced female reproduction over the duration of a breeding season.

I caught females as they approached a chorus on breeding nights and exposed them to different acoustic treatments. Females that heard conspecific calls had elevated

levels of estradiol and took longer to oviposit than control females. Levels of testosterone and progesterone were not influenced by acoustic treatment. Females oviposited regardless of whether a male was present, however, estradiol and testosterone levels were significantly elevated only in the presence of an amplectant male.

My work is the first to show the timescales over which acoustic signals may influence female physiology in frogs. Furthermore, this is the first study to demonstrate a relationship between steroids and oviposition timing in frogs. That acoustic signals influenced oviposition in an unexpected direction is a highlight of my work. Overall my research contributes to a greater understanding of the mechanisms regulating vertebrate reproduction.

Chapter 1

Hormonal modulation of phonotaxis and advertisement-call preferences in the gray treefrog (*Hyla versicolor*).

Noah M. Gordon and H. Carl Gerhardt

ABSTRACT: Hormonal levels fluctuate during the breeding season in many anurans, but the identity of the hormones that modulate breeding behavior and their effects remain unclear. We tested the influence of a combined treatment of progesterone and prostaglandin on phonotaxis, the key proceptive reproductive behavior of female anurans. First, we found that female gray treefrogs (*Hyla versicolor*) treated with progesterone and prostaglandin exhibited phonotaxis to synthetic male advertisement signals significantly more often than animals treated with ringers vehicle or uninjected controls. Responsive females had greater levels of plasma progesterone and estradiol compared to both control groups, suggesting that these steroids may be promoting phonotaxis. Second, we found that the selectivity of hormonally-induced phonotaxis in *H. versicolor* was similar to that observed in freshly captured breeding animals. Females made the same choices between acoustic signals after hormone treatments in tests of frequency, call rate and pulse rate, compared to their responses without treatment immediately after collection from the breeding chorus. The preference for a longer call was, however, significantly weaker after hormone induction of phonotaxis. Hormonally primed females were also less likely

to respond in any test and took longer to respond than did freshly collected females. Consequently, our study shows how progesterone-prostaglandin induced phonotaxis in female treefrogs influences both the quality and quantity of phonotaxis, relative to that exhibited by naturally breeding females.

Keywords: Phonotaxis; Progesterone; Prostaglandin; Estradiol; Anuran; Mate choice; *Hyla versicolor*

INTRODUCTION

Reproductive hormones play a major role in initiating and modulating mating behavior in a wide variety of taxa (e.g. Crews and Moore 2005; Moore et al., 2005; Wingfield 2005). Hormones can change the morphology of ornaments used to attract mates, the production and probability of responding to sexual signals, and other courtship behaviors. Hormones may also alter the sensitivity of sensory systems or the way that signals are perceived (e.g. Aitken and Capranica 1984; Hultcrantz et al., 2006; Penna et al., 1992; Sisneros et al., 2004), which has important implications for variability in mate choice or changes in female choosiness (Jennions and Petrie 1997). Therefore, the hormonal regulation of mating behavior cannot be fully understood without knowledge about the influence of these hormones on mate choice.

Positive phonotaxis is the orientation and movement toward a sound source (a signaler) by a receiver. In most anurans this is the primary proceptive mating behavior observed in gravid females, which approach calling males to initiate mating. Because breeding females also show this behavior in response to playbacks of conspecific calls

through loudspeakers, the acoustic signal rather than other sensory stimuli is sufficient to elicit phonotaxis. In one study of anurans the highest probability of phonotactic responses occurred when estradiol and progesterone levels were greatest (Lynch and Wilczynski 2005), suggesting that these steroids may be important modulators of anuran phonotaxis.

Progesterone and estradiol levels increase in female anurans during the period encompassing reproductive events, when phonotaxis occurs (Harvey et al., 1997; Itoh and Ishii 1990; Lynch and Wilczynski 2005; Medina et al., 2004). In the clawed frog, *Xenopus laevis*, receptive behaviors (thigh adduction, lack of a ticking vocalization) that would immediately follow phonotaxis in the wild were not induced by increases in estradiol or progesterone alone, but were induced by both together (Kelley 1982). Whether progesterone or estradiol are specifically involved in the changes in phonotaxis behavior that occur at this time is, however, unknown. Progesterone and prostaglandin injections were used together to induce phonotaxis in American toads (*Bufo americanus*; Schmidt 1985), but progesterone alone may not result in phonotaxis (Schmidt 1969, this is his assertion, data are unreported). However, Schmidt (1985) did not test uninjected females, making it difficult to determine whether phonotaxis would have also occurred in these animals without the injection of hormones.

Several lines of evidence suggest that prostaglandins are also involved in regulating phonotaxis. A combined human chorionic gonadotropin (HCG) and progesterone induction of phonotaxis can be halted with a prostaglandin inhibitor and reinstated with prostaglandin $F_{2\alpha}$ injection (Schmidt 1984), but prostaglandin alone was generally ineffective in eliciting phonotaxis (Schmidt 1985). Thus prostaglandin may be necessary but not sufficient to induce phonotactic behavior. Prostaglandin $F_{2\alpha}$ levels

increase in ovulating frogs, possibly from GnRH stimulation of oviducts (Gobbetti and Zerani 1992), suggesting its involvement in the control of oviposition, a behavior that would naturally follow phonotaxis. Consequently, prostaglandin $F_{2\alpha}$ is elevated near the time of phonotaxis and may be an important modulator of phonotactic behavior.

Phonotaxis is usually selective. Animals respond to a circumscribed set of sounds, with properties similar to those of long-range signals produced by conspecific individuals and sometimes including those of closely related species (Gerhardt and Huber 2002). In "choice" situations, however, breeding females show remarkable selectivity that not only excludes responses to the signals of other species in favor of conspecific signals but also shows discrimination among conspecific signals with subtle acoustic differences (review: Gerhardt and Huber 2002). If there is an interaction between a female's hormonal state and her reception of auditory signals, then we would predict that hormonal state could influence her phonotactic behavior and selectivity. Hormonal profiles do change seasonally in anurans (reviewed in Rastogi et al., 2005), and there is some evidence that hormonal state does influence sensory perception. Seasonal changes in midbrain auditory neuron sensitivity occur in *Hyla chrysoscelis*, the sister species of *H. versicolor*, showing that females had lower auditory thresholds during the breeding season, compared to the thresholds of frogs tested outside the breeding period (Hillery 1984). Steroids (estradiol or testosterone) have been shown to increase the evoked responses of midbrain auditory neurons (Yovanof and Feng 1983) or the number of neurons that respond in some anurans (Aitken and Capranica 1984; Urano and Gorbman 1981), but not others (Penna et al., 1992). Furthermore, estradiol has been associated with increases in gene expression in the torus semicircularis (Lynch and Wilczynski 2008) a

region of sensorimotor integration in anurans (Endepols and Walkowiak 2001). Thus, the probability of phonotaxis and its selectivity are likely to be influenced by a female's hormonal state.

Prior studies investigating the hormonal control of phonotaxis have focused on inducement of phonotaxis to a single - typically invariant - species-specific call (e.g. Boyd 1994; Kelley 1982; Picker 1983; Schmidt 1984). Alternative acoustic stimuli were used in phonotaxis studies of the túngara frog, *Physalaemus pustulosus*; however, the alternative call varied in several acoustic parameters simultaneously (Lynch et al., 2005; Lynch and Wilczynski 2005; Lynch et al., 2006). A more complete understanding of the hormonal control of anuran phonotaxis requires learning how different hormones contribute to various aspects of female selection criteria. Our subjects were gray treefrogs, *Hyla versicolor*, for which an extensive body of knowledge about phonotactic selectivity exists (Gerhardt and Huber 2002). Here we explicitly test if modulation of phonotaxis by progesterone and prostaglandin influences either the quantity (probability and number) or quality (speed and selectivity) of responses in four tests of alternative acoustic signals in which there was a single difference in the value of an acoustic property of known behavioral significance (Gerhardt and Doherty 1988; Gerhardt et al., 2000; Gerhardt 2005a, 2005b; Klump and Gerhardt 1987).

METHODS

All procedures outlined in this study were approved by the University of Missouri Animal Care and Use Committee protocol #1910. Animals were collected under Missouri Department of Conservation Wildlife collector's permits #12923 and #12343. *Hyla*

versicolor females were initially collected in amplexus from a natural breeding chorus in the Thomas Baskett Wildlife Conservation Area near Ashland, MO, USA during the 2005 (April 5-June 22) and 2006 (April 13-June 6) breeding seasons.

Acoustic Testing Procedure

We evaluated female responsiveness and selectivity by means of playbacks of synthetic advertisement calls that were generated using custom designed software (by J. Schwartz) and modified using Cool Edit (Syntrillium Co, Phoenix, AZ, USA). Our standard call was 837 ms (18 pulses) long with a pulse rate of 20 pulses/s; the spectrum consisted of two components of 1.1 and 2.2 kHz, with the amplitude of the low-frequency component 6 dB less than that of the high-frequency component. The call period was 4 s. Our standard synthetic call is equivalent to a call from an "average" male from our population (descriptions and variability of natural calls can be found in Gerhardt et al., 1996). In two-speaker tests, there was no statistically significant difference in the proportion of females choosing the standard synthetic call and pre-recorded exemplars in two-alternative, forced-choice tests (Gerhardt 1978). We tested this standard call against one of four alternative, less attractive stimuli: 1) Call Duration test - a shorter alternative call (645 ms =14 pulses); 2) Pulse Rate test - with a faster pulse-rate alternative (30 pulses/s); 3) Call Rate test - with a slower alternative call rate (8 s call period); and 4) Spectral test - with a call of higher frequency (1.4 + 2.8 kHz peaks, with the amplitude of the low-frequency component 6 dB less than that of the high-frequency component). Previous experiments showed that field-collected, gravid (or “reproductively active”) females taken from amplexus preferred the standard call to these, or similar, alternatives

(Gerhardt and Doherty 1988; Gerhardt et al., 2000; Gerhardt 2005a, 2005b; Klump and Gerhardt 1987). Every female was tested with all four of these acoustic tests, with at least 30 min separating successive tests. The order of tests was haphazard, and the speaker broadcasting the standard call was haphazardly alternated to minimize the risk of side biases, none of which were detected.

All tests were conducted in the semi-anechoic chamber described in Gerhardt (1995) at 20±1°C. For each test, females were placed in a small hardware cloth cage midway between two Analog-Digital-Systems 200 speakers that were separated by 2 m. The sound pressure level (SPL re 20 μPa, fast root-mean-square) of the stimuli was equalized at 85 dB SPL at this release point with a Larsen-Davis 800B sound level meter. Females were released by remotely removing the top of the cage after alternating stimuli from the acoustic tests described above were broadcast at least three times, with equal periods of silence between successive presentations of alternatives.

During testing, frogs were observed with a remote camera and infrared illumination. A response was tabulated when a female moved to within 10 cm of one of the speakers after showing phonotactic orientation movements, such as head and body scanning that occurred during or shortly after several calls (not necessarily each call) in a playback series (Rheinlaender et al., 1979). We also recorded the time to make a choice. A "no response" was recorded when the female failed to show phonotactic behavior within 10 min of release; some females remained in the release cage and others wandered around randomly in the chamber without showing phonotactic orientation movements.

Experiment 1: Hormonal inducement of phonotaxis

We tested female gray treefrogs (n=45) for phonotaxis, using the four acoustic choices outlined above, under three different treatments: 1) hormonal priming with progesterone and prostaglandin $F_{2\alpha}$; 2) control injections with amphibian ringers (vehicle) and 3) no injection. Our treatment of progesterone and prostaglandin (both from Sigma-Aldrich, St Louis, MO, USA) was a procedure modified from Schmidt (1985). Dosages were modified because in preliminary tests the recommended dosages for American toads (*Bufo americanus*) (Schmidt 1985) resulted in unacceptable levels of mortality and poor responses from surviving gray treefrogs. Based on Schmidt's (1985) equation of: dose-=((body mass/100 g)$^{0.666}$)*K we adjusted dosages so that K=2 mg progesterone was used (note that this is a non-linear relationship between body mass and dose, which is approximately equivalent to 36 mg/kg body mass over the range tested). This resulted in near zero mortality. We adjusted prostaglandin dosages to improve responses such that K=1200 µg (approximately equal to 21.8 mg/kg body mass over the tested range). Progesterone was injected intraperitoneally into the right medial side, posterior to the liver. Intramuscular injections of prostaglandin were administered into the thighs, with dosages divided evenly between both legs, 19±1 hours after progesterone administration. Frogs that were tested under the ringers treatment were injected with equivalent volumes of amphibian ringers at the same time as hormonally treated frogs. All frogs were tested under all three treatments with at least three weeks between treatments. The order of treatments for each frog was randomized.

Frogs tested in experiment 1 were all long-term captives that had been housed individually for greater than eighteen months. All testing was completed between

September and November of 2007. The person observing the phonotaxis tests was blind to which treatments the frogs received.

To assess how our hormonal treatment might be influencing hormonal levels, at the conclusion of testing each day a sample of blood (~100 µl) was collected from each frog via cardiac puncture. Blood samples were stored up to 24 hr at 2-8 °C and then centrifuged to separate and remove the plasma. Plasma was then stored at -20 °C until assayed. We also sampled frogs in the field approaching the breeding chorus to determine if our treatments were at physiologically relevant levels.

Hormonal analyses were done with commercial radioimmunoassay kits (Progesterone: Coat-a-Count TKPG-2, Siemens, Los Angeles, CA; Estradiol: ImmuChem 07-138102, MP Biomedicals, Orangeburg, NY). All samples were run in duplicate. Samples were diluted to 10 µl sample in 90 µl zero standard buffer prior to assay. Kits were validated using serial dilution of a pooled sample of *Hyla versicolor* plasma. Curves generated from these serial dilutions were parallel to the standard curves (data not shown). Mean intra-assay coefficients of variation for progesterone and estradiol were both 17.1% (both based on 6 standards run with each assay). Inter-assay coefficients of variation were 7.1% for four progesterone assays and 13.7% for five estradiol assays. The minimum detection limit for the progesterone assay was 0.05 ng/ml and for the estradiol assay was 10 pg/ml.

Experiment 2: Changes in selectivity in hormonally induced frogs

We tested individual female gray treefrogs both during the breeding season and after administration of hormones outside of the breeding season to compare natural

versus progesterone-prostaglandin induced phonotactic. Females were initially collected from amplexus in breeding choruses (n=109) in 2005 and 2006. After collection, females were held in coolers on melting ice for up to 7 days, and then warmed to 20 °C in an incubator prior to testing**.** All females were tested with the four phonotaxis tests outlined in the acoustic testing procedure above. The number of days between collection and testing did not affect the choices females made (see Results).

Females that responded in all four phonotactic tests during the breeding season (n=66) were toe-clipped for identification and housed in captivity until subsequent testing following hormonal priming in September - November of the same year. Hormonally primed females were acclimated overnight at 20 °C in the same incubator used for breeding season tests, and the hormonal priming and phonotactic testing procedure was the same as described above. Not all females responded in every test when hormonally primed; therefore, some females were treated as many as three times in an attempt to obtain responses for all tests. At least two weeks separated successive hormonal treatments to minimize possible carryover effects. The person recording the responses of hormonally primed females did not have knowledge of their previous responses during the breeding season.

Statistical analysis

Multiple responses from the same female to different tests are non-independent; therefore, we used Cochran's Q test to compare the proportions of females responding to different treatments in experiment 1 and to compare the responses of breeding season and hormonally primed frogs in experiment 2. To determine if the proportions of frogs

responding to the standard call were different across years or across different dates within the same year we used chi-square analysis.

We wanted to know if hormone primed frogs took the same amount of time to make choices as the same frogs tested during the breeding season, therefore we used Wilcoxon sign-rank tests to compare matched-pair responses for differences in the time to make a choice between these groups. ANOVA was used to test if there was an influence of the number of days since capture on the time to make a choice. Effects of days since capture on the probability of a female responding were analyzed with a log-likelihood test.

The distribution of hormone levels was non-normal, so levels of progesterone and estradiol were log-transformed to achieve normality prior to statistical analysis. The values reported here are, however, the untransformed values. Because each female was tested with all treatments, but not all females responded, differences in hormonal levels between treatments were analyzed with a mixed model for repeated-measures. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA), except for the Cochran's tests, which were hand calculated. Values are presented as means ± standard errors.

RESULTS

Experiment 1: Hormonal inducement of phonotaxis

Positive phonotaxis was observed significantly more often in female treefrogs treated with progesterone and prostaglandin than in females receiving either amphibian

ringers or no injections (Table 1). This result was robust, regardless of the acoustic stimulus tested (Table 1). Additionally, if we restrict our analysis to only those frogs that responded, hormone injected frogs were likely to respond in significantly more tests (2.8± 0.2 tests) compared to ringers-injected (1.4± 0.1 tests) or uninjected frogs (1.5± 0.2 tests) (n=101 frog-tests, X^2=32.79, p<0.0001). Fewer frogs responded in the ringers-injected treatment compared to the uninjected controls (Table 1), suggesting that the injections themselves had a depressive effect on female responses. Date of testing did not influence the likelihood of a female responding (n=101 frog tests, X^2=1.256, p=0.262).

Hormonally primed frogs had greater progesterone levels than ringers-injected or uninjected frogs ($F_{2, 92}$=20.88, p<0.0001) (Fig. 1-A). This indicates our injections of progesterone had the expected effect. Estradiol levels were elevated as well in hormonally primed frogs when compared to ringers-injected and uninjected frogs ($F_{2, 96}$=13.12, p<0.0001) (Fig. 1-B). Measured hormone levels of progesterone-prostaglandin treated frogs were not significantly different from wild females naturally approaching a breeding chorus (progesterone: $F_{1,43}$=2.225, p=0.1431; estradiol $F_{1,46}$=1.697, p=0.1991) (Fig. 1-A+B), so our treatments were within the natural physiological range.

The number of times a frog responded was positively correlated with both progesterone ($F_{1,91}$=7.378, p=0.0079) (Fig. 2) and estradiol levels ($F_{1,95}$=4.875, p=0.0296) (Fig. 2). Progesterone levels were positively correlated with estradiol levels in hormonally treated frogs (Fig. 3). Neither progesterone ($F_{1,95}$=0.557, p=0.447) nor estradiol levels ($F_{1,95}$=0.410, p=0.514) were influenced by the date of treatment.

Experiment 2: Changes in selectivity in hormonally induced frogs

A majority of females chose the standard call in all tests during the breeding season and after hormonal priming (Fig. 4-A). There were no significant differences between the proportion of females choosing the standard call during the breeding season and after induction of phonotaxis in tests of differences in pulse rate, frequency or call rate (pulse rate: n=33, Q=0.143, p=0.721; spectral: n=36, Q=0.077, p=0.857; call rate: n=35, Q=1.00, p=0.343). The proportion of females choosing the call of longer duration was significantly lower when females were hormonally induced than when tested during the breeding season (n=38, Q=12.00, $p<0.001$), although a statistically significant majority (24 of 38 females, binomial test: $p = 0.035$) still preferred the longer call (Fig. 4-A).

When comparing breeding season vs. non-breeding season choices, 28 of the 46 females that responded, changed their choice to the other alternative stimulus in at least one test. Nine of the 28 individuals that responded differently did so in more than one test. With the exception of the call-duration test, females that switched call preferences were as likely to switch from standard to alternative calls as they were to switch from alternative to standard calls. All 12 females that responded differently in the call-duration test switched from a preference for the standard call during the breeding season to the short-call alternative when hormonally primed.

Hormonally induced frogs took longer to respond than frogs tested during the breeding season. This difference was statistically significant for the spectral and call rate discrimination tests, and marginally significant for the test of call duration discrimination

(Fig. 4-B) (pulse rate: n=33, Z=87.0, p=0.122; spectral: n=36, Z=200.0, p=0.0009; call duration: n=35, Z=129.5, p=0.0593; call rate: n=35, Z=199.0, p=0.0005).

Females were more likely to respond phonotactically during the breeding season than after hormone treatments (Table 2). Twenty seven percent (116 of 426) of all frog tests resulted in a "no response" during the breeding season, while 51% (149 of 291) of the tests of the hormone-primed frogs resulted in a "no response." Tests of the difference in call rate resulted in fewer "no responses" when compared to the other three tests for both breeding-season and hormone-primed individuals (Table 2).

Of the 50 frogs tested after hormonal priming, twenty-four were administered hormones on multiple occasions (up to three administrations, each at least 14 days apart) in an attempt to get responses in all four tests. Of these 24 frogs, nine responded only during the first administration; four responded only after the second administration; ten responded on multiple occasions; and one never responded.

During the breeding season, 66 of 109 females responded in all four tests. The number of days between collection and testing influenced the likelihood of an individual's responding (n=109, X^2=28.41, p<0.0001) but did not influence the animal's choice (i.e. whether she chose the standard or alternate call) (n=66, pulse rate: X^2=0.36, p=0.548; spectral: X^2=0.06, p=0.810; call duration: X^2=1.00, p=0.317; call rate: X^2=0.12, p=0.729). There was also no significant effect of the number of days since collection on the time required to make a choice ($F_{4,57}$=0.758, p=0.557). Of those frogs that responded, the proportion of frogs responding to the standard call during the breeding season was not significantly different between 2005 and 2006 (n=66; pulse rate: X^2=1.72, p=0.190;

spectral: X^2=1.23, p=0.267; call duration: X^2=1.18, p=0.278; call rate: X^2=1.27, p=0.260).

To confirm that the response of female frogs to hormone treatments was consistent between years (i.e. that hormonally primed frogs of experiment 1 were comparable to those primed in experiment 2), we tested for differences in the proportion of hormonally-primed females responding to any test. There was no effect of year on the proportion of females responding after progesterone-prostaglandin treatment (n=132, X^2=2.40, p=0.301). There was also no effect of year (2005-2007) on the proportion of females choosing the standard call when hormonally primed (pulse rate: n=53, X^2=3.10, p=0.213; spectral: n=53, X^2=4.03, p=0.134; call duration: n=53, X^2=0.50, p=0.780; call rate: n=52, X^2=0.87, p=0.646)

DISCUSSION

Progesterone-prostaglandin treatment of female gray treefrogs induced phonotaxis toward playbacks of synthetic advertisement calls. Our study confirmed that this induction of phonotaxis by progesterone-prostaglandin is similar to that exhibited by naturally breeding females in terms of the selectivity of such responses to three of the four acoustic parameters tested. Females were, however, significantly less likely to choose calls of longer duration after progesterone-prostaglandin treatment, although a significant majority of treated females still chose the long-duration call. Furthermore, the probability of a phonotactic response after hormonal treatment was significantly reduced, compared to naturally breeding frogs. Because neither progesterone (Schmidt 1969, Kelley 1982) nor prostaglandin alone (Schmidt 1985) consistently resulted in phonotaxis

in previous studies, our results support the hypothesis that the combined effect of the two hormones is sufficient for eliciting this behavior in frogs.

In our study, frogs that were treated with progesterone and prostaglandin had elevated levels of estradiol, a steroid that has also been implicated in the regulation of receptive behavior (Kelley 1982). However, elevated levels of estradiol (Diakow et al., 1978; Kelley 1982) or progesterone (Kelley 1982) alone have not been shown to promote receptive behaviors in other species of anurans. Prostaglandin has been shown to increase estradiol release in post reproductive *Rana esculenta*, resulting in increased ovarian mass, but prostaglandin did not induce ovulation when administered alone (Gobbetti et al., 1990). This observation suggests that prostaglandins could be responsible for the increased estradiol levels noted here. Progesterone alone may also contribute to the observed increase in estradiol through its metabolism into estradiol, or it could be promoting estradiol release or synthesis through another pathway. We noted an increase in estradiol after our hormonal administrations, raising the possibility that estradiol might mediate the influence of these hormones on the phonotaxis we observed. Because Kelley (1982) found that administration of progesterone and estradiol together was needed to induce the receptive behaviors that naturally follow phonotaxis, our hormonal treatment may be effectively equivalent to hers. Further work will be needed to confirm this hypothesis and to learn whether the progesterone, prostaglandin, or an interaction of the two is responsible for the estradiol increase. Additionally, while we can conclude that our progesterone-prostaglandin treatment induced the observed phonotaxis behavior, we can not determine whether it was the progesterone, prostaglandin, estradiol, or some combination thereof that ultimately caused this behavior.

Steroid levels in breeding frogs may increase in wild frogs via gonadotropins. HCG has been shown to increase plasma estradiol in a manner consistent with naturally phonotactic females (Lynch et al., 2006) and may be responsible for increases in progesterone as well (Morrill et al., 2006; Thornton 1972). Both *Xenopus laevis* (Picker 1983) and *Physalaemus pustulosus* (Lynch et al., 2006) respond to HCG injections with phonotactic behavior. However, since estradiol levels did not change across the stages of reproduction when phonotaxis would occur in *R. esculenta* (Gobbetti and Zerani 1992), changes in estradiol may not be necessary or sufficient for eliciting phonotaxis in all species of anurans. Of course, some minimum level of estradiol may still be needed to induce this behavior.

Females of *H. versicolor* usually made the same choices between acoustic signals when their phonotaxis was induced by injections of progesterone and prostaglandin as they did after being collected in a breeding chorus and tested within a few days. As expected from the results of previously published studies (Gerhardt 2005a, 2005b; Gerhardt and Doherty 1988; Klump and Gerhardt 1987), females preferred the standard call in both conditions. Hormonally induced females did show a significantly weaker preference in the call-duration test than when tested shortly after capture; however, a majority of females still preferred the longer call. The weakening of a preference for call duration is noteworthy. Gray treefrog females use call duration as a cue to evaluate male quality (Gerhardt et al., 1996). Choosing longer calling males has been shown to convey genetic benefits for traits that improve offspring survival (Welch et al., 1998); consequently, a weakening of this preference could have implications for reproductive fitness.

Hormonally primed females were also less likely to respond in any test and usually took longer to respond than did freshly collected females. Similar results were found in comparisons of phonotaxis in *P. pustulosus* captured during the breeding season (Lynch et al., 2005) with that of hormonally primed (with HCG) post-mating animals (Lynch et al., 2006): there was no obvious difference in the proportion of females responding to the species-typical call, though this was not explicitly tested.

Our combination of progesterone-prostaglandin (modified from Schmidt 1985; see Methods) was usually effective in inducing phonotaxis in post-reproductive females. Administration of HCG or arginine vasotocin (AVT) has also been shown to induce phonotaxis in female anurans (Boyd 1994; Kelley 1982; Lynch et al., 2006; Picker 1983; Schmidt 1985). While the relationships between the hormones that may be involved in female anuran reproduction are not yet well understood, there is some suggestion that the actions of both HCG and AVT may overlap or coincide with the actions of progesterone and/or prostaglandin. Gonadotropins increase prostaglandin production in anuran interrenal glands (Gobbetti and Zerani 1991), ovaries, and oviducts (Gobbetti and Zerani 1992) during the reproductive period. Gonadotropins also increase progesterone production in the follicles during the same period (Chang et al., 1997; Kwon et al., 1993). Furthermore, both AVT-induced (Diakow and Nemiroff 1981) and HCG-induced (Weintraub et al., 1985; Schmidt 1984) receptive behaviors can be inhibited by a prostaglandin inhibitor. Our results may thus prove to be similar to hormonal induction of phonotaxis using these other hormones, although this awaits further confirmation.

Mature ovarian follicles and oviposition were not required for the observed induction of phonotaxis in gray treefrogs. We have successfully used this protocol with

three different species in the gray treefrog complex (*Hyla versicolor, H. chrysoscelis* and *H. arenicolor*) in every season and in almost every month of the year, and oviposition is only occasionally observed (Gordon and Gerhardt, unpublished). The reduction in phonotactic responses in other anurans that occurs when HCG is used to induce this behavior may be a result of insufficient numbers of well-developed follicles, as suggested by Picker (1983) to explain why only about 30% of the females of African clawed frogs, *Xenopus laevis*, responded after injections of HCG. The fact that all female *Xenopus* induced to phonotactic receptivity with HCG oviposited in a separate study (Weintraub et al., 1985) corroborates this assertion. Lynch et al., (2006) inferred that only females of *P. pustulosus* with mature follicles respond to HCG treatment, though this was not explicitly tested. *P. pustulosus* breeds repeatedly throughout much of the year (Ryan 1985), while the hylid treefrogs we studied have a narrower period of reproductive activity. Though it is possible that these North American *Hyla* maintain mature follicles throughout the year, our protocol does not appear to depend on females having eggs in the same state as in the breeding season.

Numerous studies have used hormones to elicit reproductive behavior such as vocalization, amplexus, and phonotaxis in frogs and toads (e.g. Boyd 1994; Gerhardt et al., 1994; Kelley 1982; Noble and Aronson 1942). These studies have either sought to discover causal relationships between hormones and mating behavior (e.g. Lynch et al. 2006; Weintraub et al., 1985) or used hormonal treatment as a practical means of assessing variation in mating behaviors among individuals, populations, or species under the same environmental conditions (e.g. Gerhardt 1994; Gerhardt et al., 1996). The latter application implicitly assumes that the selectivity of individuals under treatments that

induce such behavior in the laboratory reflects natural conditions. Here we have shown that these behaviors are, in fact, similar.

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Table 1

The percent of frogs exhibiting phonotaxis in four tests of acoustic selectivity for hormonally injected (n=38), vehicle (ringers) injected (n=38), and untreated females (n=42). The percent shown reflects the total number of frogs responding, regardless of whether they chose the standard or alternative call.

	% of frogs responding to calls:				
Treatment:	Un-injected	Ringers	Progesterone/ prostaglandin	Cochran's Q	*p*
Pulse rate	7	8	53	25.185	<0.001
Spectral	12	3	45	18.995	<0.001
Call duration	12	5	39	14.709	<0.001
Call rate	10	3	45	20.667	<0.001
All tests combined	26	13	66	19.185	<0.001

Table 2

Percent of tests resulting in a "no response" (frogs that did not approach either stimuli within 10 min) for breeding season and the same females hormonally primed after the breeding season.

Test	% of breeding season tests resulting in "no response"	% of hormone primed tests resulting in "no response"
Pulse rate	32	58
Spectral	30	54
Call duration	33	47
Call rate	10	43
All tests combined	27	51

Fig. 1. Plasma progesterone (A) and estradiol (B) levels for frogs treated with progesterone and prostaglandin, amphibian ringers (vehicle), uninjected controls and wild-caught breeding females. Both plasma progesterone and estradiol levels were significantly greater in progesterone-prostaglandin treated frogs, relative to the same frogs under the ringers or uninjected control treatments (both $p<0.0001$). Hormone levels of progesterone-prostaglandin treated females were not significantly different from field-collected animals (progesterone: $p=0.1431$; estradiol: $p=0.1991$). Values are presented as mean ± SE with sample sizes above each bar.

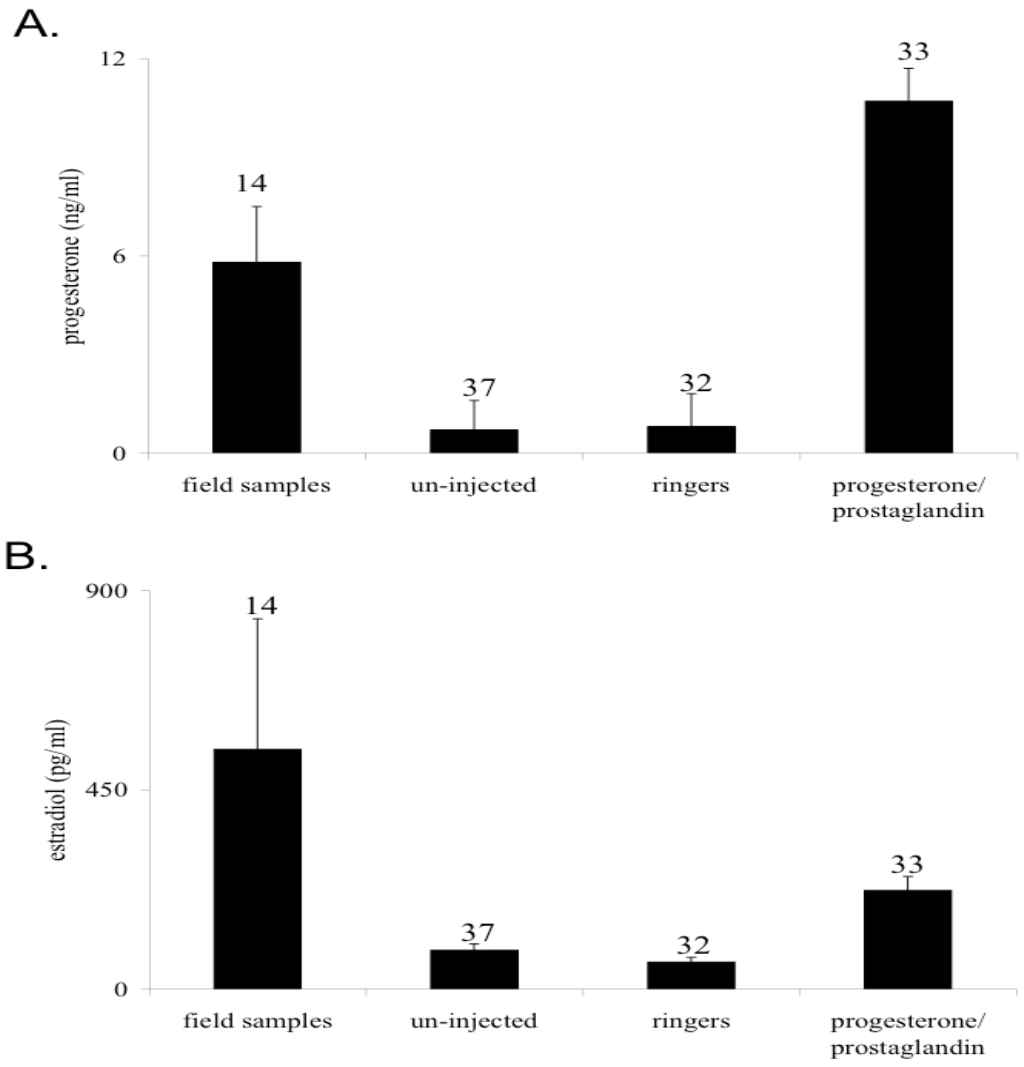


Fig. 2. Mean (±SE) plasma progesterone and estradiol levels as a function of the number of tests in which a frog responded regardless of treatment. Females that responded to more tests had greater progesterone (p=0.0079) and estradiol (p=0.0296) levels.

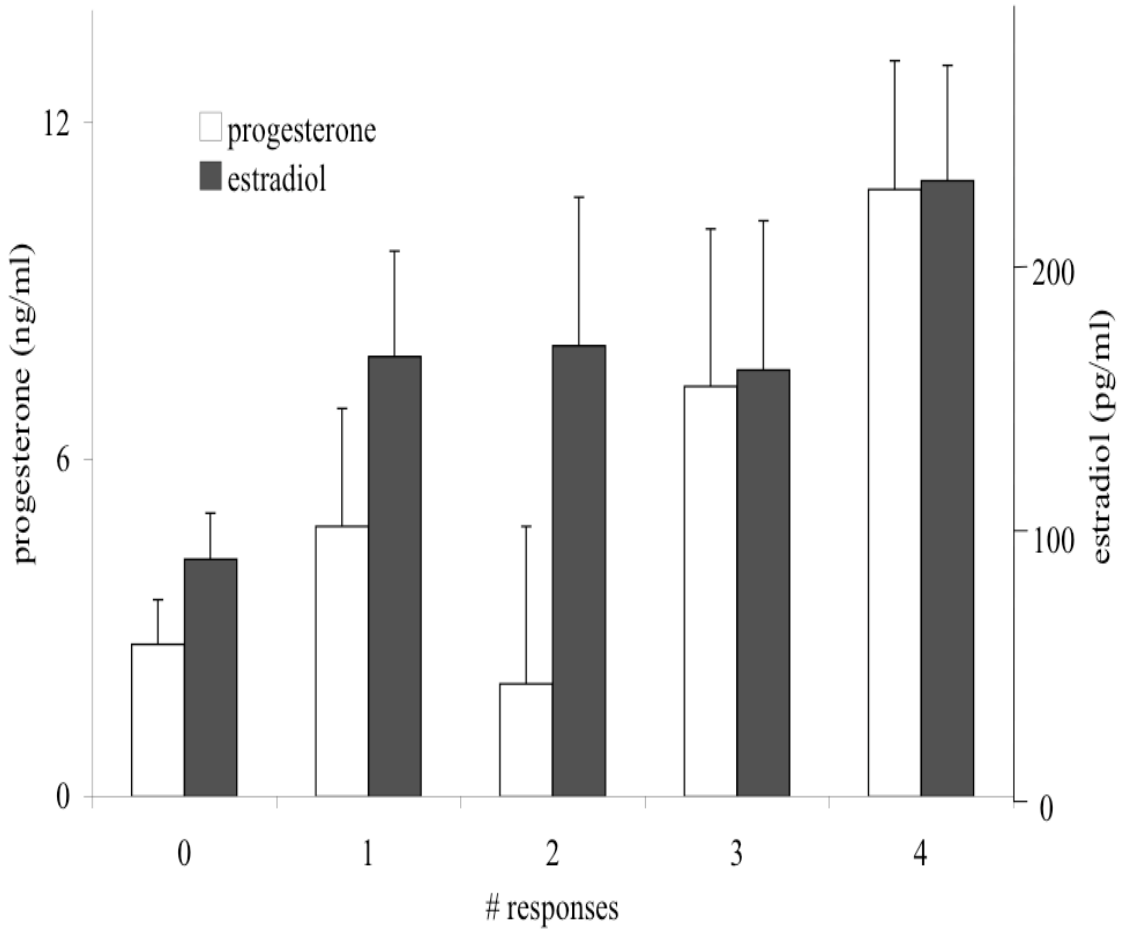


Fig. 3. Relationship between plasma progesterone and estradiol concentrations in female frogs injected with progesterone and prostaglandin.

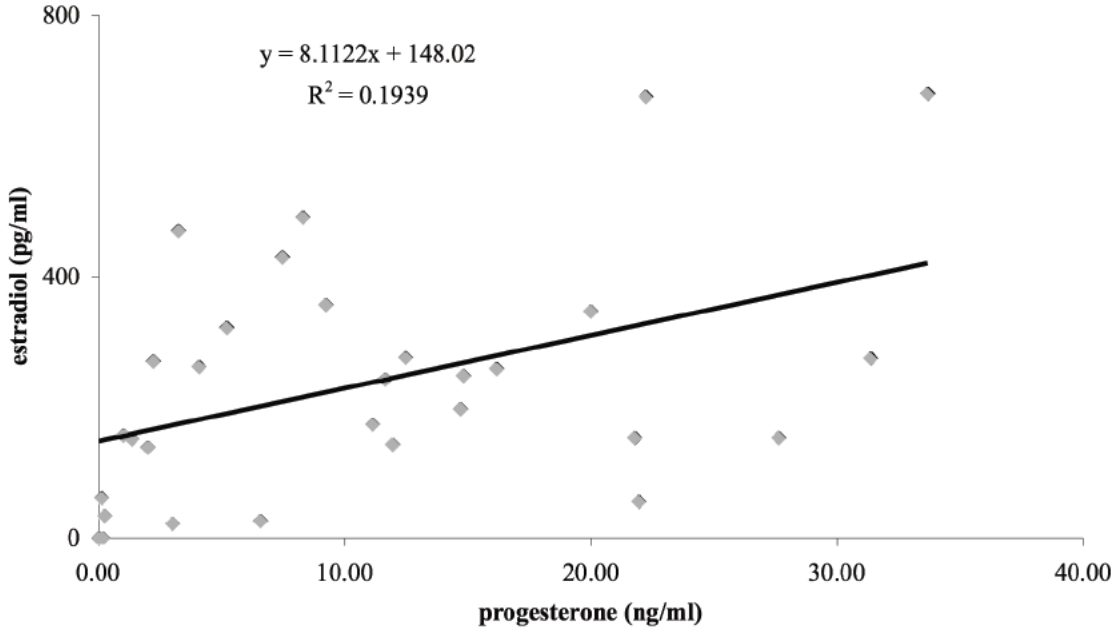
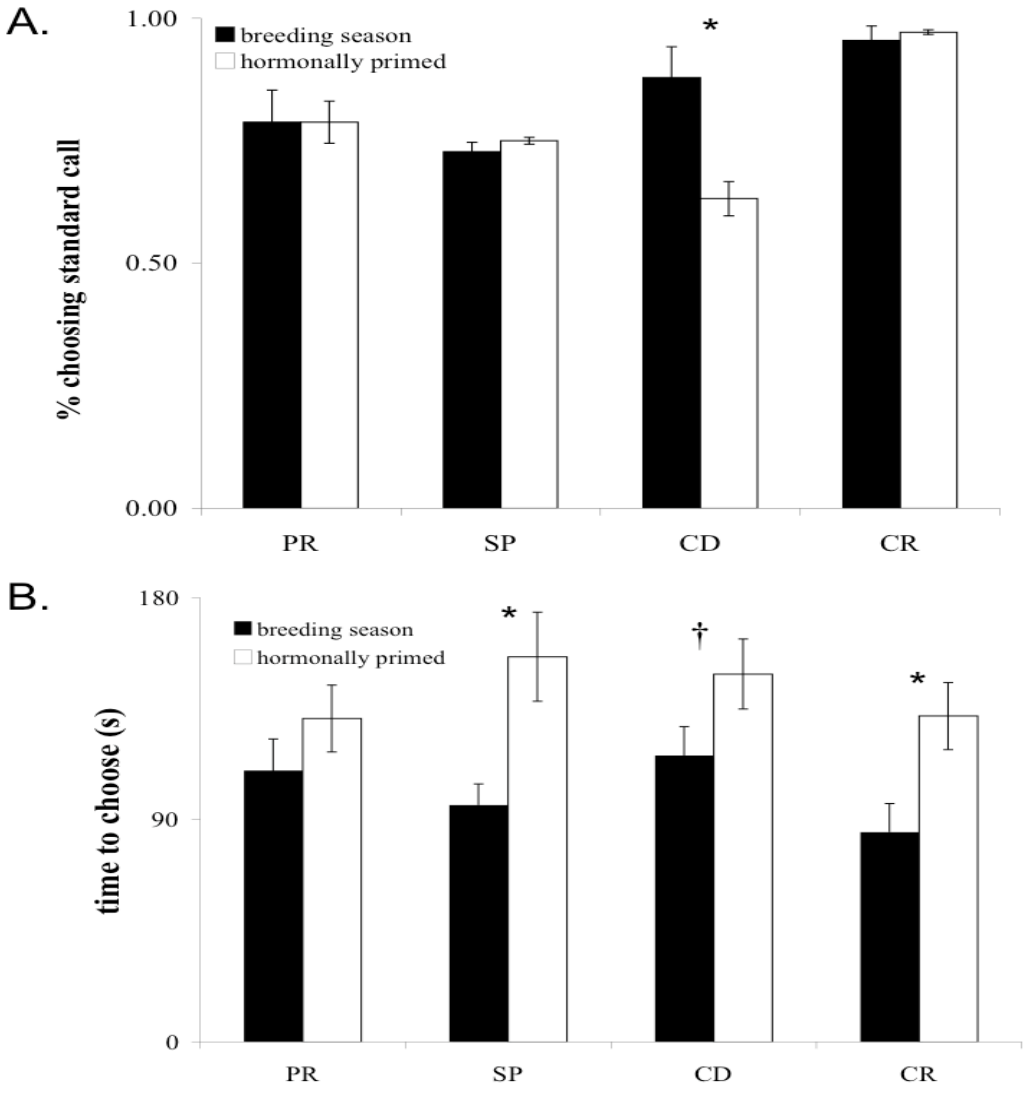


Fig. 4. (A) Comparison of the choices made during the breeding season by untreated females and by the same females following progesterone-prostaglandin treatment. (B) Comparison of the time required to make a choice by untreated females tested during the breeding season and the same females following progesterone-prostaglandin treatment. PR=pulse rate test, SP=spectral test, CD=call duration test, CR=call rate test, *=significant at p≤0.001. †=marginally significant at p=0.059.



Chapter 2

Gonadal steroids and body condition in a prolonged breeding anuran: seasonal and breeding night variation in females.

Noah M. Gordon and Michelle Hellman

ABSTRACT: The patterns of hormones that influence reproduction in prolonged-breeding, multiple-clutching anurans remain unclear. We used field sampling of gray treefrogs, *Hyla versicolor*, to measure steroid hormone levels of breeding and non-breeding frogs throughout the active seasons for this species. Gray treefrog females showed the greatest elevation of all measured steroids on breeding nights, and non-breeding females had greater plasma estradiol and testosterone during the breeding season relative to the non-breeding season, suggesting that these frogs exhibit an associated pattern of reproduction. Annual patterns of testosterone and estradiol were similar, suggesting a relationship between these hormones that may be indicative of prolonged-breeding, multiple-clutching anurans. While progesterone levels were greatest on breeding nights, there was no relationship between progesterone levels and season. Females captured during the breeding season were in poorer condition compared to those captured after the season, though body condition was not correlated with reproductive steroid levels. Our findings suggest that either steroids are not good

predictors of breeding activity, or that reproductive activity is not strongly condition dependent in this species.

INTRODUCTION

The endocrine system plays an important role in the regulation of amphibian reproductive behavior. More specifically, the hypothalamic-pituitary-gonadal axis controls the release of gonadal steroids, which influence the timing of an organism's reproductive behavior. Three relationships between the level of gonadal steroids and behavior have been described. In the most common pattern, the associated pattern (sensu Crews and Moore 1986), steroid levels are elevated at the same time as reproductive behavior is exhibited. In the dissociated pattern, steroid levels are elevated when behaviors are not exhibited (Crews and Moore 1986). In the constant pattern, steroid levels are elevated for prolonged periods, regardless of behavior (e.g. Bentley et al., 2000).

Gonadal steroid levels increase in female anurans typically during the period encompassing reproductive events (Harvey et al., 1997; Itoh and Ishii 1990; Lynch and Wilczynski 2005; Medina et al., 2004). Gonadotropins may cause increases in steroid levels in breeding frogs. For example injections of human chorionic gonadotropin (HCG) have been shown to increase plasma estradiol to levels typical of females that show phonotaxis to conspecific calls (Lynch et al., 2006); HCG may also be responsible for increases in progesterone (Morrill et al., 2006; Thornton 1972).

The gray treefrog, *Hyla versicolor*, is an arboreal frog found throughout Eastern North America. Except for the nights when breeding occurs and during winter

brummation, females occur in trees up to several hundred meters from breeding ponds (Johnson et al., 2007). While females may breed several times during a prolonged breeding season that can last several months, each breeding event lasts only one night. For at least some females, breeding activity commences soon after emergence from brummation, and the breeding season can last for up to four months. The behavior and location of females after the breeding season is indistinguishable from that of non-breeding females during the breeding season (Johnson et al., 2007, this study).

The cues that trigger breeding for individual female gray treefrogs are poorly understood. Warm spring days and rainfall are associated with the beginning of the breeding season, but at least some females in a population can be found breeding on almost every night thereafter for a period of several months. Although most females oviposit only once per year, two clutches are common, and up to five clutches in a single season have been reported (Roble 1985 for the sister species *H. chrysoscelis*). A gap of at least 3-4 weeks occurs between clutches for females that breed multiple times (Humfeld and Gordon pers. obs.; Roble 1985 for the sister species *H. chrysoscelis*). The ability to oviposit more than one clutch in a season and the likelihood of reproducing early in the following year, are probably both more dependent on the foraging success of the female (Ritke and Lessman 1994) than on seasonal or environmental cues (Alexander and Bellerby 1938).

Changes in hormone levels may influence neurological centers that control reproductive behaviors. In *H. chrysoscelis*, the sister species of *H. versicolor*, evoked potentials of neurons change seasonally in the torus semicircularis (Hillery 1984) - a primary site of sensorimotor integration (Endepols and Walkowiak 2001). Hillery (1984)

hypothesized that these differences in evoked potentials may have resulted, in part, from changes in the reproductive state of the treefrogs and seasonal fluctuations in hormone levels. Seasonal hormonal changes have not yet been measured in the gray treefrog complex, so this remains an intriguing hypothesis.

The first objective of our study was to determine levels of steroid hormones associated with reproduction in *H. versicolor*, both within and outside of breeding events in order to learn what reproductive pattern this species exhibits (sensu Crews and Moore 1986). We will show that female gray treefrogs likely exhibit an associated reproductive pattern, and we will provide evidence about which steroids likely influence reproduction in this species. Our second objective is to determine if differences in the body condition of frogs found during and outside the breeding season are correlated with differences in hormone levels. We will show that non-breeding females captured during the non-breeding season are in better condition than females captured in the breeding season, and that body condition is not correlated with reproductive steroid levels.

METHODS

All procedures outlined in this study were approved by the University of Missouri Animal Care and Use Committee protocol #1910. Animals were collected under Missouri Department of Conservation Wildlife collector's permits #12343 and #13232. All sampling was done at the Thomas Baskett Wildlife Conservation Area near Ashland, MO, USA during 2006 (April - October) and 2007 (March - October). We occasionally checked for frogs on warm days (ambient temperature > 15 C) in late autumn and winter (November - March), but none were observed outside of the aforementioned dates. Our

sampling periods thus encompass the entire non-brummation period for *H. versicolor* at this site during the period studied. We defined the breeding season at our site to include all the months in which choruses formed (>5 males) at the breeding pond and mating was observed. All females captured in arboreal retreats (see below) at least 20 m from breeding choruses were considered non-breeding, regardless of season. Females only breed at night, consequently we only sampled for non-breeding females during daylight hours.

Sampling of terrestrial habitat for non-breeding females

PVC pipes (Johnson 2005) were attached to tree trunks at a height of 1.5-2 m to simulate water filled tree-hole refugia. Pipes were placed at approximately 20 m intervals at distances up to 300 m from the breeding pond in two rows, in each of the four cardinal directions for a total of 100 pipes. Each row in a cardinal direction was at least 20 m from the nearest row. Generally the capped bottom end of the pipe contained a few centimeters of rainwater, but we occasionally added aged tap water during dry periods to encourage frog occupancy.

Pipes were checked for the presence of frogs twice weekly. We recorded the mass, snout-vent length, sex, date of capture and distance from breeding pond for all captures. Frogs that were less than 5 g were arbitrarily considered juveniles and were returned to the pipe of capture without further sampling. Frogs greater than 5 g were toe-clipped for identification and a sample of blood (<100 μl) was taken via cardiac puncture for hormonal analysis. Some frogs were captured repeatedly over the course of this

study, generally in the same pipe; however, blood samples were not taken more than once per month from the same individual.

Sampling of breeding females

On breeding nights with active choruses female gray treefrogs were captured at one of two stages of reproduction: prior to chorus arrival as females approached the breeding pond; or paired with males in amplexus. Blood samples were taken as above at one of these reproductive stages. Frogs were toe-clipped for identification to prevent multiple sampling of the same frog.

Hormonal analysis

Blood samples were returned to the laboratory within 3 hr, stored up to 24 hr at 2-8 °C and then centrifuged to separate and remove the plasma. Plasma was then stored at -20 °C until assayed.

Hormonal analyses were done with commercial radioimmunoassay kits (Progesterone and Testosterone: Coat-a-Count, Siemens, Los Angeles, CA; Estradiol: ImmuChem, MP Biomedicals, Orangeburg, NY). All samples were run in duplicate. Samples were diluted to 10 µl sample in 90 µl zero standard buffer prior to assay. Kits were validated using serial dilution of a single pooled sample mixed from several frogs. Curves generated from these serial dilutions were parallel to the standard curves generated for each hormone (data not shown). The testosterone kit has 3.3% cross reactivity with dihydrotestosterone. Mean intraassay coefficients of variation for testosterone were 11.2%, progesterone were 9.1% and for estradiol were 11.6%.

Interassay coefficients of variation were 14.4% for two progesterone assays and 16.1% for three estradiol assays. All testosterone samples were done in a single assay. The minimum detection limit was 0.1 ng/ml for the progesterone assay, 0.2 ng/ml for testosterone and 10 pg/ml for estradiol. Some plasma samples were not sufficiently large enough to perform assays for all three steroids so sample sizes are not always equivalent.

Statistical analysis

We did not have enough recaptures of non-breeding females to do a repeated-measures analysis across the two years of this study, and sample sizes were too small to test for differences between months in measured steroid levels. Consequently we pooled our results by only including the first captures in each month that were independent of previous months, and used one way ANOVA to compare steroid levels during the breeding season to those outside of the breeding season. For illustrative purposes we included all samples (i.e. both independent and repeated measures) in figure 2B, but reported statistics are based on the independent values only.

The ability to reproduce may be influenced by female foraging success (Ritke and Lessman 1994). Consequently, we assessed the condition of treefrogs to test for its influence on measured steroid levels. We used the residuals from a regression of mass on snout-vent length as a measure of condition. Morphological measurements from several studies over several years (Gerhardt et al., unpubl.) were combined for our population to improve the explanatory power of this regression. The linear fit from this regression explained most of the observed variation (N=133, adjusted $R^2 = 0.747$) (Fig. 1). We then excluded all individuals with residuals within +/- 0.5 standard deviations of the mean,

and classified all remaining individuals with a positive residual as being in good condition, and all individuals with a negative residual as being in poor condition. Chi-square analyses were used to determine if the proportions of frogs in good condition were different between groups.

RESULTS

Annual variation in non-breeding females

We caught 31 females in our pipes a total of 133 times over the two years of this study. Of these 133 frog-captures, 31 recaptures were separated by at least one month. Consequently we had 62 plasma samples over the two years of this study, but only 31 independent samples. Each recaptured frog was found in the same pipe in which it was found the first time.

Non-breeding female treefrogs had significantly greater plasma estradiol ($F_{1,29} = 5.542$, $p = 0.0255$) and testosterone ($F_{1,26} = 5.991$, $p = 0.0214$) if they were captured during the breeding season (March-June) than if they were caught outside of the breeding season (July-October). There was no difference between plasma progesterone during and after the breeding season ($F_{1,29} = 0.177$, $p = 0.678$) (Fig. 2).

Non-breeding females were significantly more likely to be in better condition during the non-breeding season than those captured during the breeding season ($N= 15$, $\chi^2= 3.864$, $p=0.0493$) (Fig. 3). There was no relationship between body condition and hormone levels for non-breeding females captured during the breeding season (estradiol:

N=9, t = 0.059, p = 0.9543; progesterone: N=9, t = 0.716, p = 0.4974; testosterone: N=9, t = -0.281, p = 0.7865) (Fig. 4), however, sample sizes were small.

Non-breeding females were not found closer to the pond during the breeding season than during the non-breeding season ($F_{1,29} = 0.0331$, p = 0.8568) (Fig. 5). For non-breeding females, there was no influence of distance from the pond on plasma steroid levels, regardless of season (estradiol: $F_{1,29} = 0.1148$, p = 0.7372; progesterone: $F_{1,29} = 0.2566$, p = 0.6163; testosterone: $F_{1,26} = 0.0591$, p = 0.8098) (Fig. 6). There was also no relationship between the distance a treefrog was captured from the breeding pond and her condition (N= 14, t = 0.3169, p=0.7575) (Fig. 7).

Breeding night variation

Levels of all steroids measured were greatest as females approached the chorus to breed. Testosterone ($F_{1,10} = 9.223$, p = 0.0125) was significantly greater in pre-amplexed frogs than in those found in amplexus, while both estradiol ($F_{1,18} = 0.511$, p = 0.4840) and progesterone ($F_{1,15} = 2.581$, p = 0.129) levels were greater in the pre-amplexed group than in the amplexed group, but not significantly so (Fig. 8).

Hormone levels for frogs caught at choruses on breeding nights were significantly greater than hormone levels in non-breeding frogs caught in pipes (estradiol: $F_{1,45} = 21.24$, $p < 0.0001$; progesterone: $F_{1,46} = 11.92$, p = 0.0012; testosterone: $F_{1,38} = 23.29$, $p < 0.0001$).

DISCUSSION

We have shown that female gray treefrogs have the greatest levels of plasma steroids (progesterone, estradiol and testosterone) as they approach a pond to breed. Moreover, non-breeding females captured during the breeding season had greater plasma testosterone and estradiol levels than did frogs captured outside of the breeding season; however, these levels were not significantly less than those of actively breeding frogs. These results are consistent with the associated reproductive pattern of elevating reproductive steroids during breeding events, which is typical of vertebrates (Crews and Moore 1986). While we cannot rule out a constant reproductive pattern (Crews and Moore 1986), some non-breeding females had no measurable steroids during the breeding season. Finding such low steroid levels would support the constant reproductive hypothesis only if these females did not breed that entire season, which we regard as highly unlikely. Female gray treefrogs do not exhibit a dissociated reproductive pattern because levels of all measured hormones were lowest outside the breeding season. A dissociated pattern would be unexpected because such a pattern is usually associated with harsh climates and/or sporadic resource availability (Crews and Moore 1986).

Non-breeding female treefrogs occur, on average, further from ponds than males, and females probably only approach ponds on the night of breeding (Johnson et al., 2007). If frogs move closer to breeding ponds when breeding events are imminent, we would expect that females found closer to breeding ponds would have greater levels of circulating hormones than frogs found further away. We did not find a relationship between steroid levels and distance from the breeding pond, an observation supporting

the hypothesis that female reproduction in this species is a relatively rapid event. Hormonal surges, ovulation and oviposition probably all occur within a span of a few days. This concurrence of hormone levels with reproduction provides further support for an associated reproductive pattern in this species.

Annual hormone patterns

Annual population-level changes in specific hormones may reflect breeding activity, possibly reflecting individual variation in ovarian development. However, it remains unclear if population-level changes in specific hormone levels in prolonged breeders are reflective of breeding activity. This may be caused in part by individual variation in ovarian development and by variability between species in the hormones associated with reproductive behavior. Although there are exceptions, in many anurans annual progesterone peaks generally predict breeding activity (Rastogi et al., 2005). For example, *Leptodactylus ocellatus*, a subtropical anuran from South America, maintains high progesterone levels throughout its prolonged breeding season without an obvious peak even though other steroids such as estradiol and androgens are more cyclical (Mosconi et al., 1996). Seasonal levels of androgens and estradiol are often correlated with oviduct and ovarian weight (Rastogi et al., 2005). We did not find a significant elevation in progesterone levels coinciding with the breeding season in gray treefrogs, though levels were greatest on breeding nights. This observation suggests that progesterone may play a permissive role in breeding but it is not a good predictor of breeding activity in *H. versicolor*. Differences in the timing of ovarian, oviduct, or

follicle development, as well as differences in breeding phenology probably all contribute to the observed differences in hormonal profiles between species.

The annual pattern of serum concentrations of estradiol and testosterone was similar across the two years of this study. Both hormones were elevated during the breeding season and reduced afterwards (Fig. 2). Concurrent annual patterns of these two steroids been found in only two other species: *Pachymedusa dacnicolor* (Iela et al., 1986) and *Rana esculenta* (Pierantoni et al., 1984). *H. versicolor* (Gordon and Humfeld unpubl.), *P. dacnicolor* (Bagnara and Rastogi 1992; Bagnara et al., 1986) and *R. esculenta* (Pierantoni et al., 1984) are all prolonged breeders (sensu Wells 1977) that produce multiple clutches in a single season. Both *P. dacnicolor* (Iela et al., 1986) and *H. chrysoscelis* (Ritke and Lessman 1994), the sister species of *H. versicolor* - also a prolonged breeder with multiple clutches - maintain follicles in multiple stages of development throughout a breeding season, which could be a mechanism for the parallel relationship of these two steroids (Kwon et al., 1993). The genus *Rana* is distantly related to the other two hylid genera (*Hyla* and *Pachymedusa*) (Frost et al., 2006). Thus, it remains an intriguing possibility that there may be a relationship between this annual parallel pattern of testosterone and estradiol levels and prolonged-breeding multiple-clutching anurans.

Role of steroids in reproduction

Estradiol may have several roles in anuran reproduction. Physiologically it has been shown to promote vitellogenesis and follicular growth in other anurans (Bruscalupi et al., 1998; Pierantoni et al., 1987), so it is likely to have a similar role in gray treefrogs.

There is also increasing evidence that estradiol may have a role in promoting phonotaxis behavior (Gordon and Gerhardt 2009; Kelley 1982; Lynch and Wilczynski 2005) - a behavior that is required for reproduction to occur in most anurans. Changes in estradiol may, however, be insufficient for eliciting reproductive behaviors in all species of anurans. Estradiol levels do not change across the stages of reproduction when breeding occurs in *R. esculenta* (Gobbetti and Zerani 1992), suggesting that the role of estradiol may be permissive, rather than activational. This influence of estradiol on behavior may be the result of an interaction with progesterone (Gordon and Gerhardt 2009; Kelley 1982).

In addition to playing a role in promoting reproductive behaviors (Gordon and Gerhardt 2009; Kelley 1982), increased levels of progesterone on breeding nights may be responsible for promoting nuclear maturation of the oocytes and, ultimately, fertilizable gametes (Jalabert et al., 1991; Ramos et al., 2001). Progesterone has also been shown to play a role in oviduct development and jelly secretion (Pierantoni et al., 1984).

While the physiological roles of testosterone in anurans are not as clear, there is evidence for its involvement in promoting oviduct growth (Dubowsky and Smalley 1993), oocyte maturation (Le Goascogne et al., 1985; Ramos et al., 2001), and vitellogenesis in the liver (Di Fiore et al., 1998). There is also indirect evidence for a role of androgens in ovulation (Morrill and Bloch 1977).

Influence of body condition

Some authors have assumed that only females above some minimum threshold of condition will reproduce (e.g. Ritke and Lessman 1994, Alexander and Bellerby 1938).

We did not find a relationship between body condition and steroid levels in non-breeding females captured during the breeding season. Lack of a correlation between steroid levels and body condition reflect a lack of influence of body condition on reproduction in this species. Another possibility is that steroid levels are simply not good predictors of impending reproduction in this species. We favor the latter hypothesis, though because our sample sizes were small we can not rule out the possibility that condition does influence reproduction. Of course it is also possible that all frogs in our study area were in good condition relative to other populations.

A surprising result of our study was that significantly fewer non-breeding females (37.5%) were in good condition during the breeding season, compared to outside the breeding season, when 85.7% were in good condition. Measures of body size were not found to predict the quantity of food ingested in gray treefrogs (Mahan and Johnson 2007), so it does not appear that larger females eat more at each feeding, or eat more because they are larger. Non-breeding season captured females may, however, be feeding more frequently to maintain their larger size. This difference in condition suggests several non-exclusive hypotheses. One possibility is that current events regulate condition. In this scenario, food intake remains constant during the females active period, but the potentially greater energetic demands of reproduction decrease average body condition during the breeding season. Alternatively, food abundance could be driving this observed condition difference. Food supply, and not climatic cues have been shown to be the primary regulator of reproductive timing in captive *Xenopus laevis* (Alexander and Bellerby 1935, Alexander and Bellerby 1938, Bellerby 1938). Foraging success has also been suggested to influence the timing of reproduction in *H.*

chrysoscelis, the sister species of *H. versicolor* (Ritke and Lessman 1994). A third possibility is that females may feed more during the non-breeding season to build energy stores to promote overwintering survival (as suggested by Layne et al., 1998), or to promote future reproductive events. Further work will be needed to test these non-exclusive hypotheses relating reproduction and feeding.

Females captured farther from breeding ponds tend to have greater stomach content mass than those captured near breeding ponds (Mahan and Johnson 2007). However, we found no relationship between distance from the breeding pond and a female's body condition. If our condition index is reflective of the actual robustness of the frogs in our study, then females further from breeding ponds may be eating larger meals less frequently. The sample sizes in both Mahan and Johnson (2007) and this study are small, so further sampling should be undertaken to confirm this hypothesis.

Conclusion

We have shown that female gray treefrogs exhibit an associated reproductive pattern with the greatest levels of plasma steroid hormones just prior to breeding events. Annual patterns of testosterone and estradiol were similar, suggesting a relationship between these hormones that may be indicative of prolonged-breeding, multiple-clutching anurans. We have also shown that females captured during the non-breeding season were in better condition compared to those captured during the breeding season, suggesting that energetic demands may be greatest during the breeding season.

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FIGURES

Fig. 1: Relationship between mass (g) and snout-vent-length (SVL, in mm) for treefrogs from Baskett Wildlife Refuge. The equation of the linear fit is: mass = -18.71 + 0.589*SVL, n=133 females, adjusted $R^2 = 0.747$.

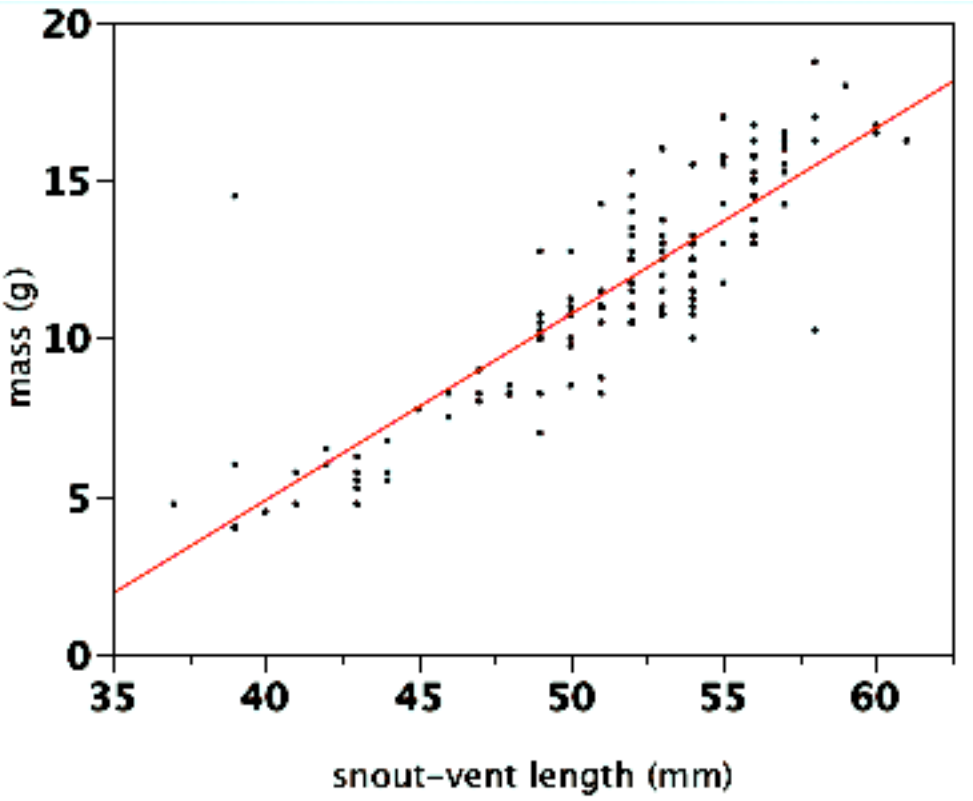


Fig. 2: A) Comparison of steroid hormone levels for non-breeding females captured within and outside of the breeding season (March-June). B) Monthly variation of steroid hormones. We did not have enough recaptures of non-breeding females to do a repeated-measures analysis across the two years of this study, and independent sample sizes were too small to test for differences between months in measured steroid levels. Consequently, we include all samples (i.e. both independent and repeated measures) in part B for illustrative purposes, but reported statistics in part A are based on the independent values only.

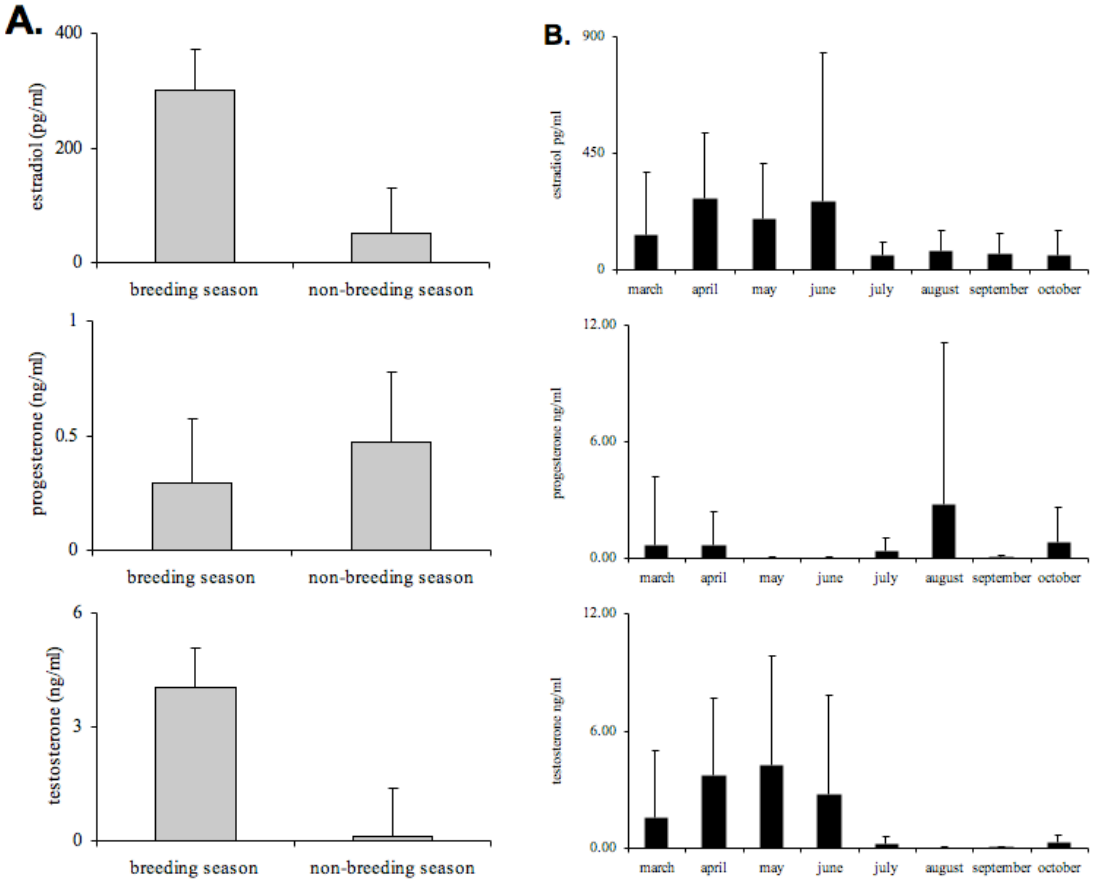


Fig. 3: Seasonal comparison of the proportion of non-breeding females that were in good (above-average) condition.

Fig. 4: Steroid levels for non-breeding female treefrogs in good and poor condition.

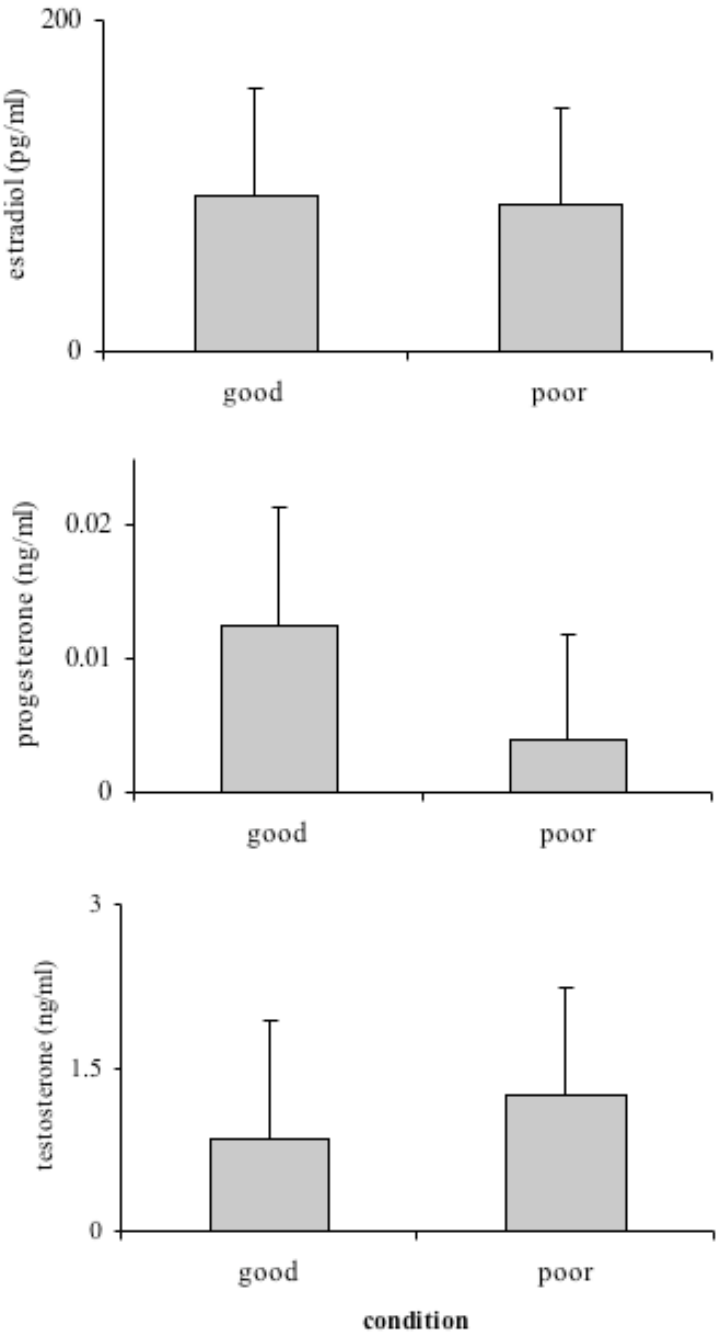


Fig. 5: Difference in capture distance for non-breeding females during and after the breeding season.

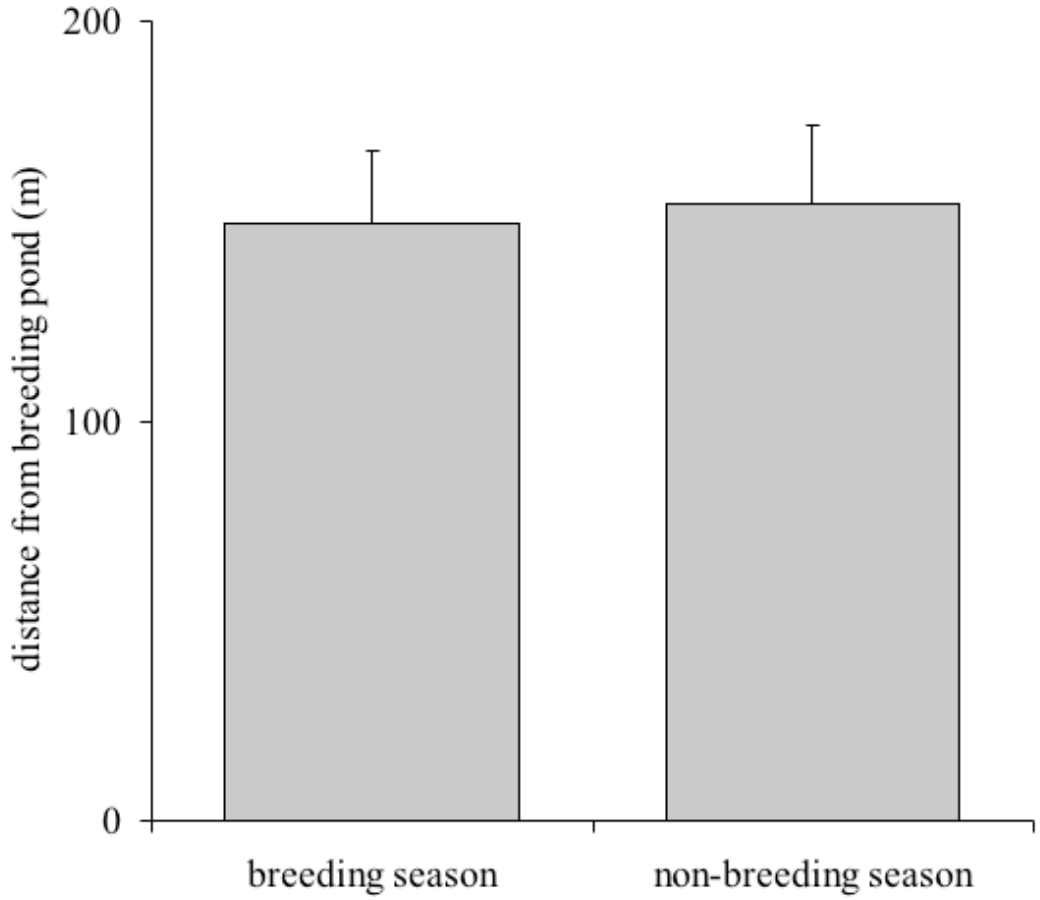


Fig. 6: Relationship between steroid levels and distance of capture (in meters) for non-breeding female treefrogs regardless of capture date. All relationships are non-significant. E2=estradiol, P4=progesterone, T=testosterone.

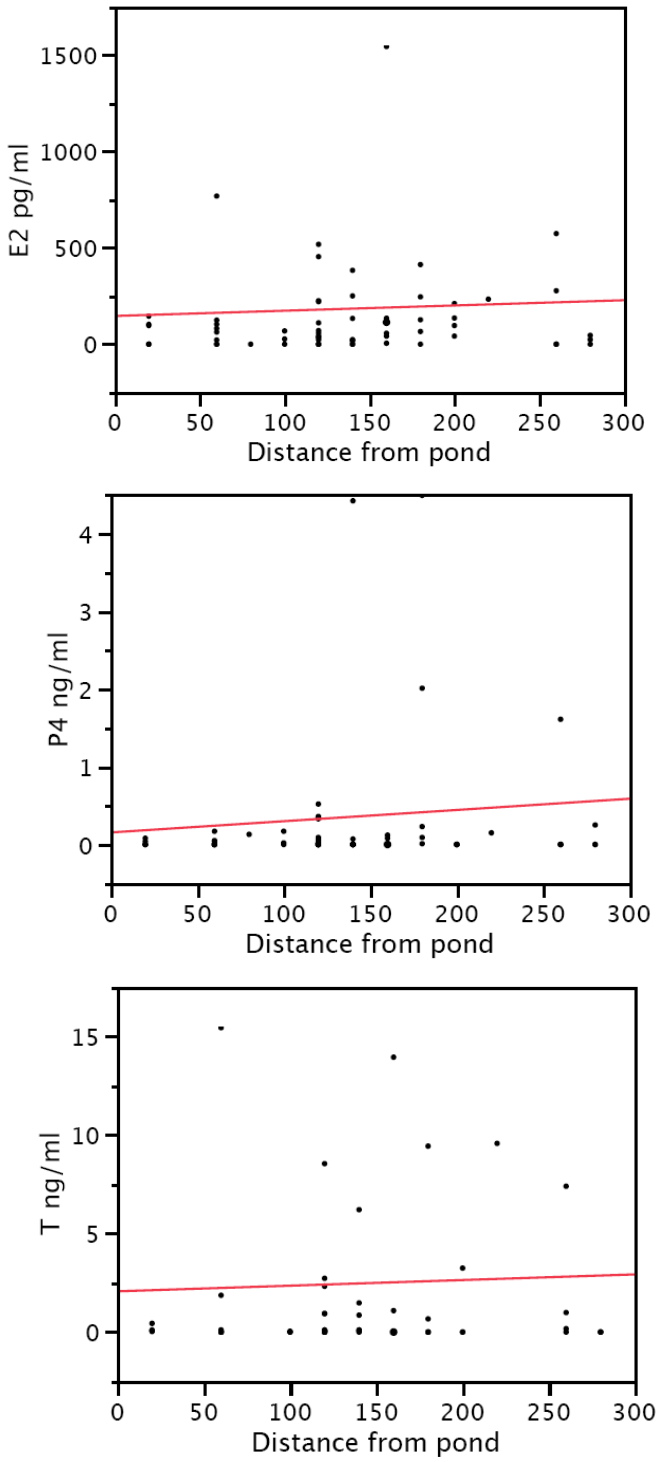


Fig. 7: Relationship between body condition and distance of capture (in meters) for non-breeding female treefrogs regardless of capture date.

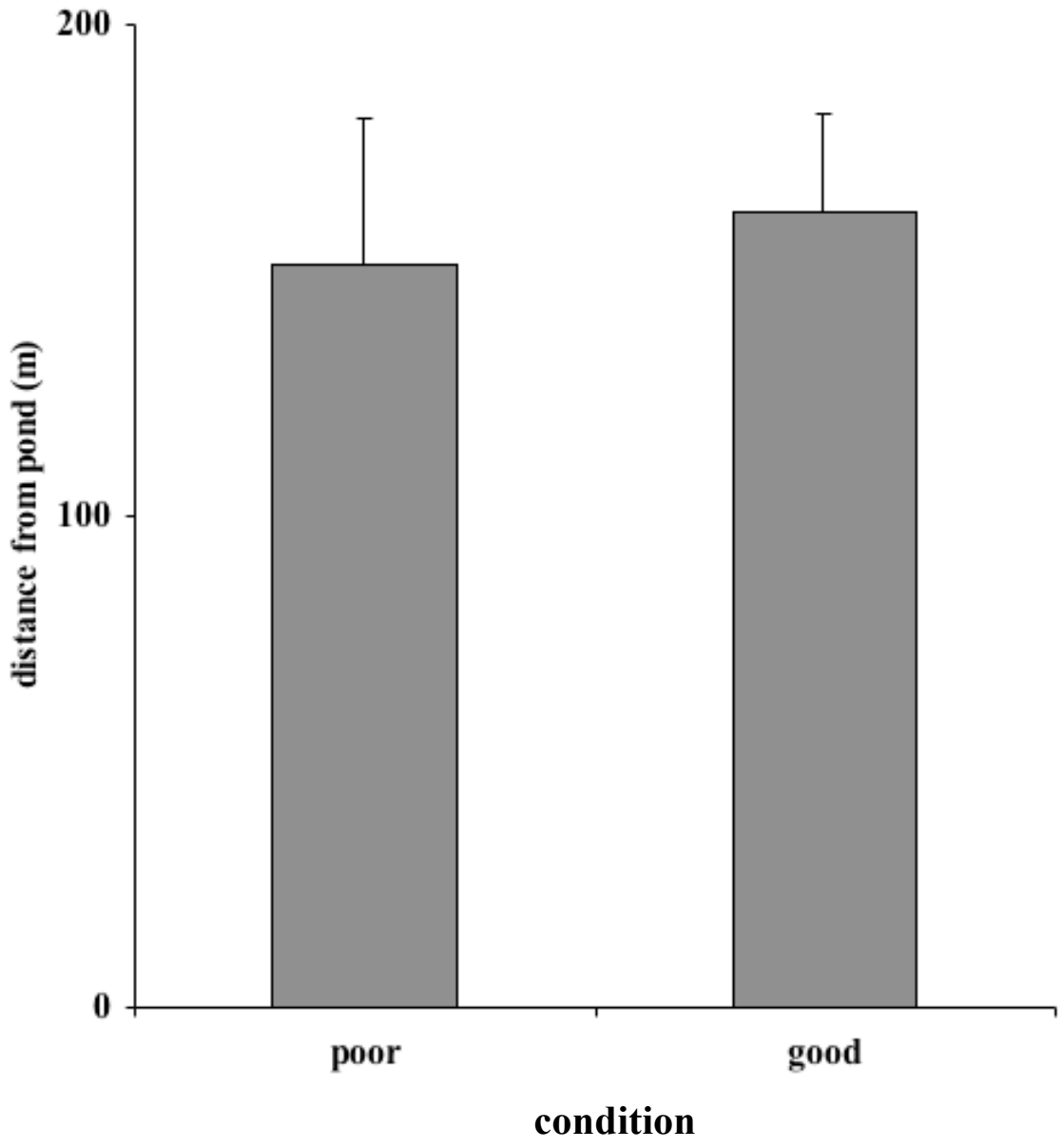
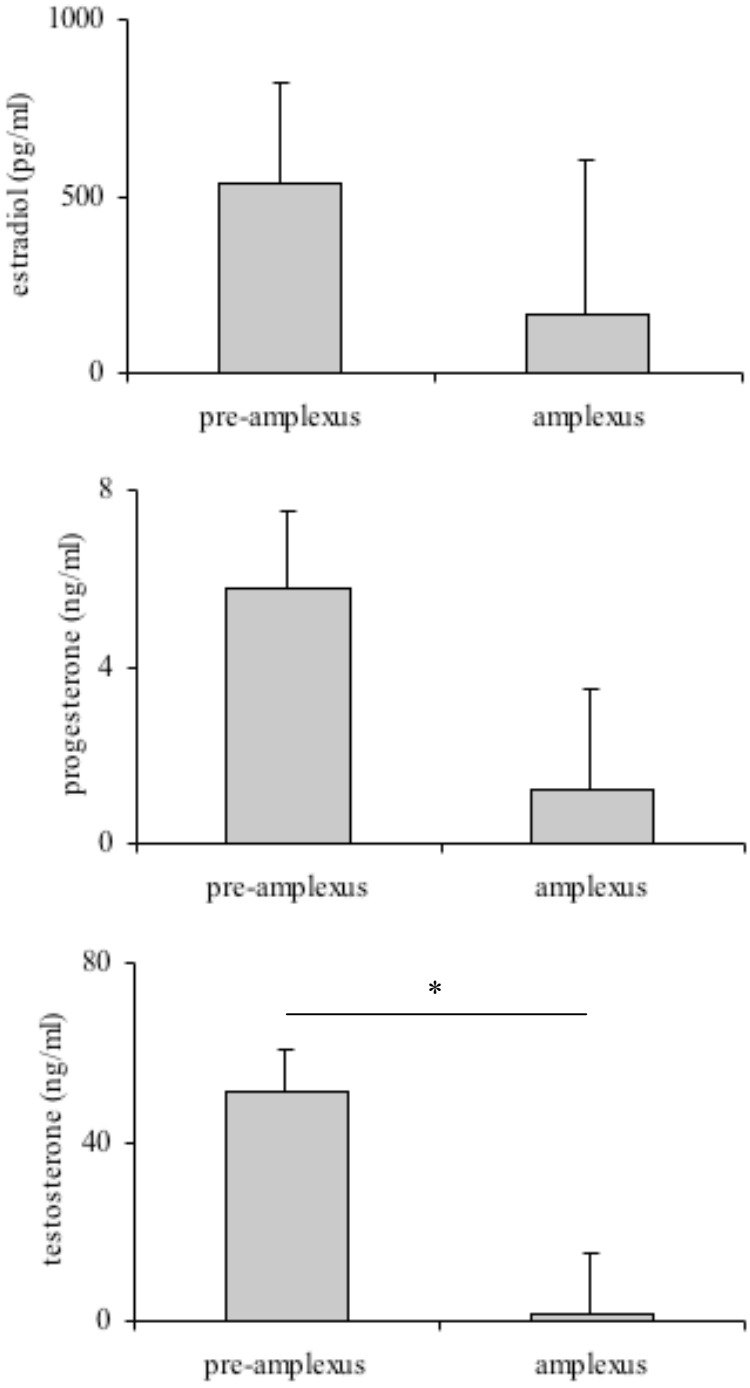


Fig. 8: Comparison of hormone levels for breeding females captured approaching a chorus and in amplexus. *= significantly different at p = 0.0125.



Chapter 3

Acoustic and amplexus-related social signals influence reproductive steroids and oviposition in female treefrogs.

Noah M. Gordon and H. Carl Gerhardt

ABSTRACT: Steroid hormones regulate reproduction and influence behaviors, but how hormones respond to exogenous cues is poorly understood. We investigated how the presence of an amplectant male and acoustic social signals interact to influence the hormones that regulate female reproductive behavior in the gray treefrog, *Hyla versicolor*. We tested the hypotheses that advertisement calls (an acoustic signal) and the presence of an amplectant male advance oviposition by influencing steroid hormone levels. Female treefrogs that heard conspecific signals had elevated plasma estradiol relative to females that heard pink noise or silence, and females had elevated estradiol and testosterone when ovipositing with an amplectant male. Progesterone levels were unaffected by either call reception or amplexus. To our knowledge this is the first conclusive evidence of amplexus acting as a social cue influencing hormone levels in anurans. We have also shown that females took longer to oviposit when exposed to conspecific signals regardless of whether a male was present for oviposition. Consequently, our data do not support the long-supported hypothesis that social

stimulation hastens reproduction, since we have shown oviposition took longer in the presence of acoustic stimulation.

INTRODUCTION

In many animals hormones have been shown to regulate reproductive cycles and influence reproductive behaviors (e.g. Vandenbergh and Drickamer 1974, Crews and Moore, 1986). While previous workers have shown correlations between hormone levels and reproductive behaviors across years or breeding seasons (e.g. Wingfield *et al.* 1997; Kim *et al.*, 1998), the cues triggering these changes remain poorly understood.

The exogenous cues that trigger female reproduction are varied. Possible cues could be environmental (e.g. day length, humidity or temperature), physiological (e.g. fat stores), or behavioral (e.g. physical mate contact or sexual advertisement signals) (Herman, 1992). Conspecific acoustic signals can act as a cue regulating reproduction: male roaring can advance the date of oestrus in female red deer, *Cervus elaphus* (McComb, 1987), and ring doves, *Streptopelia risoria*, that hear their own vocal signals show increased ovarian development (Cheng, 1992). Therefore it seems likely that vocal signals could also rapidly act to influence reproductive states in female frogs. Female Mallorcan midwife toads (*Alytes muletensis*) maintain reproductive condition and do not reabsorb their eggs in the presence of male calls (Lea *et al.*, 2001). Because oocyte maturation and re-absorption is controlled by reproductive steroids (Lin and Schuetz 1983 and 1985), the findings of Lea *et al.* (2001) strongly suggest that calls are influencing hormonal levels in this species. Female túngara frogs increase their estradiol

levels in response to male signals (Lynch and Wilczynski 2006) in a manner similar to that observed in wild females (Lynch and Wilczynski 2005). There is mounting evidence that this estradiol increase may promote anuran phonotaxis (Gordon and Gerhardt in press; Chakraborty and Burmeister, in press), and it may play a role in other reproductive events as well.

Oviposition occurs soon after phonotaxis in most frogs, so it is possible that this behavior may also be influenced by the observed changes in hormones in response to social stimulation. Darling (1938) proposed that social stimulation would hasten oviposition, and this has since been confirmed in birds (Kroodsma 1976; Waas et al., 2005). It remains untested if this behavioral phenomenon occurs in anurans.

Amplexus, the physical clasping of the female by the male, could act as a social cue influencing hormonal changes as well. The relationship between hormones and amplexus remains unclear in frogs. Several studies have shown that steroid levels change in both male and female frogs in amplexus (e.g. Harvey et al., 1997; Gobbetti and Zerani 1999; Orchink et al., 1988). However, in other anuran species steroid levels show no change after amplexus (e.g. Wada et al 1976; Licht et al., 1983). In male Japanese toads, *Bufo japonicus*, amplexus causes increases in gonadotropins, which suggests that increases in androgens may be the result and not the cause of amplectant behavior in this species (Ishii and Itoh 1992). To our knowledge no studies have conclusively determined if amplexus itself is the cue triggering these hormonal changes when they are observed. Here we tested the hypothesis that amplexus influences reproductive steroids independently, or in concert with acoustic stimulation. We will show that both acoustic stimulation and amplexus influence steroid levels and oviposition timing in gray treefrogs

(*Hyla versicolor*) but that the effect of stimulation by conspecific advertisement calls is not in the expected direction.

METHODS

All procedures outlined in this study were approved by the University of Missouri Animal Care and Use Committee protocol #1910. Animals were collected under Missouri Department of Conservation Wildlife collector's permits #12343 and #13232. Gray treefrog, *Hyla versicolor*, females were initially collected as they approached a breeding chorus using a drift fence in the Thomas Baskett Wildlife Conservation Area near Ashland, MO, USA during the months of April and May in 2006 and 2007. All frogs were captured at least 20 m from the breeding chorus and immediately placed in an insulated cooler to minimize further exposure to ambient sound. Chorus noise was audible at ground level where frogs were captured migrating to the pond, but sound pressure level (SPL re 20 μPa, C-weighting, fast root-mean-square) was low (approximately 45-60 dB SPL). Males arrive at choruses before females, and the few males that began chorusing at distances greater than 20 m from the pond (i.e. behind or on our drift fence) were moved to the pond prior to female arrival. Females tended to arrive at the fence in groups, and the time of capture for each female was recorded. Experiments were only conducted on nights when 9 or more females could be collected.

All females were brought to our facilities at the University of Missouri and immediately placed individually in cages (30 x 18 x 18 cm) with mesh tops and aged tap water to a depth of 4 cm at 22±1°C. The total time between capture, transport, and experimental set up took 2-3 hours, which included a 30 m period of acclimation in the

test cage. Females were then exposed to one of three stimuli broadcast from portable CD players (Audiovox DM8220B, Hauppauge, New York, USA) and amplifiers (Radio Shack SA-10, Fort Worth, Texas, USA) through omni-directional speakers (T.I.C. GS10, City of Industry, California, USA). The SPL of the stimuli was equalized at the cage front with a Radio Shack 33-2055 sound level meter. Females were assigned to treatments using a randomized block design, with each night constituting a block. To maintain acoustic isolation between treatments, treatments were haphazardly assigned to three separate rooms each night.

The three acoustic treatments were pink noise, silence, and a combination of conspecific call and chorus noise (see Fig. 1 for oscillograms and spectrograms of these stimuli). Pink noise was generated using Cool Edit (Syntrillium Co, Phoenix, AZ, USA). Pink noise was used rather than white noise or random tones because the SPL of pink noise declines logarithmically with increasing frequency, mimicking the sensitivity of vertebrate auditory neurons, and hence all auditory receptors would be equally stimulated. Frogs exposed to the silent treatment were tested with an unpowered speaker where background sound was approximately 40 dB SPL or less and shielded with anechoic foam. The call and chorus stimulus consisted of a synthetic "average" male call 837 ms (18 pulses) long with a pulse rate of 20 pulses/s; the spectrum consisted of two components of 1.1 and 2.2 kHz, with the amplitude of the low-frequency component 6 dB less than that of the high-frequency component. The call period was 4 s. In two-speaker tests, females responded as often to this standard synthetic call as they did to pre-recorded exemplars (Gerhardt 1978). This synthetic call was added to a recording of chorus noise from the pond where females were collected, and adjusted to be 6 dB greater

than the chorus noise. The call was synthesized using custom designed software (by J. Schwartz) and modified using Cool Edit (Syntrillium Co, Phoenix, AZ, USA). The call and chorus noise stimuli was broadcast at 83 dB SPL at the cage front during the synthetic call portion of the stimuli, which is the approximate SPL of male calls from our study population at 1 m. The pink noise stimulus was set to 79 dB SPL. We used different sound pressure levels for the pink noise and conspecific calls in an attempt to approximately equalize the total energy over time of the two stimuli, because pink noise has a constant SPL over time, whereas the call and chorus noise stimuli has variable SPL over time. We used a Larson Davis SPL meter (800B, Provo, Utah, USA) to measure and calibrate stimuli SPL over a longer time window (slow RMS setting: one second), and then measured the calibrated signals with the Radio Shack SPL meter to determine the experimental fast RMS setting.

Stimuli were broadcast continuously for 3 hours, a period approximately equal to the average duration of peak chorusing for gray treefrogs (Ritke et al., 1990; NMG pers. obs.). Frogs were checked every 30 minutes under dim light for evidence of oviposition both during and after stimuli broadcast. We recorded the time that the first egg was observed in the water, but did not consider the female to have oviposited unless >10 eggs were eventually visible. Most females oviposited several hundred eggs. We recorded the time to oviposition as the number of minutes between collection at the drift fence and the first egg of oviposition. If oviposition was not observed by 0900 (i.e. approximately 10 h after chorus arrival), we considered the female not to have come to the chorus for breeding, and excluded her from all further analysis. Twenty-eight of 113 females were excluded in this manner, and 27 of these 28 had still not oviposited after 36 h.

To assess how our treatments might be influencing hormonal levels, a sample of blood (~100 µl) was collected from a subset of females at three stages during the experiment: at the point of collection, after exposure to the acoustic stimuli, or after oviposition. Samples were taken from each frog via cardiac puncture. No frog was sampled more than once. To exclude the possibility of a sampling effect, we did not include oviposition data from frogs sampled prior to oviposition in our analysis, though all frogs sampled in this manner eventually oviposited before our 0900 cut off time. Blood samples were stored up to 24 hr at 2-8 °C and then centrifuged to separate and remove the plasma. Plasma was then stored at -20 °C until assayed.

Hormonal analyses were done with commercial radioimmunoassay kits (progesterone and testosterone: Coat-a-Count TKPG-2 and TKTT-1, Siemens, Los Angeles, CA; estradiol: ImmuChem 07-138102, MP Biomedicals, Orangeburg, NY). All samples were run in duplicate. Samples were diluted to 10 µl sample in 90 µl zero standard prior to assay. Kits were validated using serial dilution of a single pooled sample mixed from several frogs. Curves generated from these serial dilutions were parallel to the standard curves generated for each hormone (data not shown). Mean intraassay coefficients of variation for testosterone were progesterone were 6.6% and for estradiol were 15.7% (all based on 5 standards run with each assay). Interassay coefficients of variation were 22.5 % for two testosterone assays, 29.1% for two progesterone assays and 17.4% for three estradiol assays. The minimum detection limit was 0.1 ng/ml for the progesterone assay, 0.2 ng/ml for testosterone and 10 pg/ml for estradiol. The testosterone kit has 3.3% cross reactivity with dihydrotestosterone. We were not able to

obtain blood samples from all animals, and some blood samples were too small to assay all three steroids; consequently, sample sizes are not always equivalent.

To assess whether the lack of amplexus influenced hormone levels or oviposition time, we repeated the above experiment but allowed a randomly selected male to amplex the female after the three hour stimuli broadcast. We accomplished this by taking a male in amplexus (either with a female or another male) and sliding the focal female between the male and his partner until he adjusted his grasp to the focal female. This allowed us to place females into amplexus, without her hearing the calls of the male with which she was paired. The amplexed pair was then returned to the focal female's cage. Pairs that separated after this induced amplexus (and before oviposition) were excluded from all analyses.

Statistical analyses

The ability to reproduce may be influenced by female foraging success (Ritke and Lessman 1994). Consequently, we assessed the condition of treefrogs to test for its influence on the likelihood of females ovipositing. We used the residuals from a regression of mass on snout-vent length as a measure of condition (see Chapter 2, Gordon and Hellman for details of this regression), and used Chi-square analysis to test for an influence of this residual on oviposition probability.

We tested whether our acoustic treatments influenced the proportions of females that oviposited with contingency table analyses. To test for an influence of the acoustic stimuli on hormone levels after the three hour broadcast period we used one-way ANOVA. We expected that there might be an interaction of amplexus with our acoustic

treatments or the time to oviposit for frogs sampled after oviposition. Therefore we used ANOVA to test for effects of acoustic stimulus, amplexus, time to oviposition and all two-way interactions on steroid levels. If these whole models were significant we used Tukey, F- or t-tests on subsets of these parameters to determine where there were differences in the effects.

RESULTS

All females used in our experiments had an equal likelihood of ovipositing. The influence of a female's condition ($N=105$, $X^2=2.203$, $p=0.1549$), or the stimuli she heard ($N=113$, $X^2=0.733$, $p=0.6933$) (Fig. 2) on her likelihood of oviposition was not significantly different from random.

The time to oviposit was influenced by the acoustic stimuli in un-amplectant females: they took longer to oviposit if they heard calls and chorus noise compared to the other treatments ($F_{2,48}=6.541$, $p=0.0031$). The pattern was the same in amplexed females, however, it was not statistically significant ($F_{2,32}=1.694$, $p=0.1999$) (Fig. 3). Power analysis suggests that doubling our sample size would have allowed us to detect significant differences for amplexed females if they were present at $p=0.05$. There was no significant difference in the time it took amplexed or un-amplexed females to oviposit ($N=63$, $t=-1.084$, $p=0.2843$).

Differences in the elapsed time prior to oviposition might be expected to influence hormone levels by allowing more time for steroid catabolism or metabolism. However, we found no relationship between time and steroid levels for two of the hormones measured (estradiol: $F_{1,56}=1.186$, $p=0.2808$; progesterone: $F_{1,55}=0.0629$, $p=0.8029$).

Although we did find a significant positive relationship between testosterone and elapsed time prior to oviposition ($F_{1,52}$=5.832, p=0.0193), the explanatory power of this regression was weak (adjusted R^2=0.0835) (Fig. 4).

In pre-amplexed (post stimuli exposure) females estradiol levels were elevated in those females exposed to calls and chorus noise relative to the other two control treatments. This difference in estradiol levels was marginally significant (One-way ANOVA $F_{2,26}$=3.124, p=0.0608; call-chorus vs pink noise: $F_{1,17}$=8.241, p=0.0106; call-chorus vs silence: $F_{1,18}$=2.012, p=0.1732). There was no difference in testosterone or progesterone levels among the three treatments (testosterone: $F_{2,19}$=0.4253, p=0.6596; progesterone: $F_{2,21}$=0.1914, p=0.8272) (Fig 5).

Post-oviposition levels of estradiol and testosterone were influenced by the presence or absence of an amplectant male, but not by the acoustic stimuli, or by the interaction of amplexus and acoustic stimuli (whole models: estradiol: $F_{5,62}$=14.005, p<0.0001; testosterone: $F_{5,58}$=6.178, p=0.0001). Progesterone levels were not influenced by any of these aforementioned factors (whole model: $F_{5,60}$=0.6221, p=0.6835). Estradiol (N=68, t=1.999, p<0.0001) and testosterone (N=64, t=2.002, p<0.0001) levels were greater in females that had oviposited while in amplexus, compared to those that were not in amplexus during oviposition (Fig. 6).

DISCUSSION

We have shown that both acoustic stimulation and the presence of an amplectant male influence the timing of oviposition and reproductive hormone levels in breeding treefrogs. Female treefrogs that heard conspecific signals had elevated estradiol levels

relative to females that heard pink noise or silence, and females had elevated estradiol and testosterone when ovipositing with an amplectant male. To our knowledge this is the first conclusive evidence of amplexus (or a cue strongly associated with amplexus) acting as a cue influencing hormone levels in female anurans. We have also shown that females took longer to oviposit when exposed to conspecific signals regardless of whether or not a male was present to fertilize eggs.

Wilczynski and colleagues (2005) hypothesized that gonadal hormones would decline more slowly across a breeding season in the presence of acoustic stimulation. Our data support this hypothesized pattern for estradiol, albeit over a shorter time scale (a single breeding night as opposed to an entire breeding season). Elevation of hormones in response to social stimulation has been noted once before in frogs: estradiol levels increased (and androgen levels did not change) in túngara frogs, *Physalaemus pustulosus*, after exposure to conspecific acoustic stimulation (Lynch and Wilczynski 2006). We found this same pattern in hormonal responses, suggesting this response may be common in acoustically communicating frogs.

Elevation of estradiol in response to conspecific signals is a transient event in gray treefrogs: after oviposition any influence of acoustic treatment on hormone levels was no longer detectable. Using acoustic signals to elevate steroids has been hypothesized to be a mechanism for promoting the maintenance of reproductive condition for future breeding events (Wilczynski et al., 2005; Lynch and Wilczynski 2006). If acoustic signals served to promote maintenance of reproductive condition through steroid elevation, then we would expect that steroid levels would decline less or more slowly after reproductive events in animals exposed to conspecific signals.

Because hormone levels in all gray treefrog females declined to similar levels after oviposition regardless of acoustic experience, we suggest that acoustic stimulation is not promoting maintenance of reproduction beyond breeding events, at least through hormonal elevation. It is still possible that acoustic signals influence the likelihood of future reproduction via a non-hormonal mechanism, or that the observed temporary hormonal change triggers some other physiological event to promote future reproduction, but these hypotheses will require further testing. It is also possible that more acoustic stimulation, beyond the three hour window we tested, might have a more prolonged effect. In many species of frogs (including *Hyla versicolor*), a subset of males call sporadically throughout the day and night during the breeding season, so a more prolonged period of stimulation does occur in nature.

Estradiol elevation could benefit both males and females by promoting phonotaxis. There is increasing evidence that estradiol plays a role in receptive behavior in many frog species (Kelley 1982; Chakraborty and Burmeister in press; Gordon and Gerhardt in press). Because the period after acoustic stimulation and before amplexus is the time when phonotaxis and other receptive behaviors are most required for reproduction, elevation of estradiol during this time interval could be most beneficial for promoting reproduction. There is evidence in the túngara frog that receptivity is maximal during the period after acoustic stimulation (Lynch et al 2005), which coincides with estradiol elevation in this species (Lynch and Wilczynski 2005). Several hormones are elevated prior to amplexus in wild gray treefrogs (Gordon and Hellman chapter 2), so this remains an intriguing hypothesis.

Influence of amplexus on steroids

Amplexus, or a cue associated with amplexus, is acting as a cue influencing hormonal changes in female gray treefrogs. Females had significantly more plasma testosterone and estradiol if they oviposited in amplexus than if there was no male present during oviposition. To our knowledge this is the first evidence that amplexus is a cue influencing hormonal changes in female anurans. It is unlikely that amplexus acts as a trigger for hormonal changes in all species of anurans. Some steroids are elevated in amplexed female frogs in all species in which this has been examined (e.g. Harvey et al., 1997; Gobbetti and Zerani 1999; Itoh and Ishii 1990). However, there is considerable interspecific variation in which steroids are elevated during amplexus. For example, the Algerian toad, *Bufo mauritanicus*, has the highest levels of testosterone when amplexed (Siboulet 1981). In contrast, female water frogs, *Rana esculenta*, show the opposite pattern: testosterone levels are lowest in amplexus (Gobbetti and Zerani 1999). Some of the variation in hormonal changes between species could reflect variation in amplectant behaviors exhibited by females (perhaps better characterized as suppression of male rejection behaviors). Estradiol and progesterone act to suppress vocalizations and leg extension in unreceptive *Xenopus laevis* (Kelley 1982), whereas these steroids did not repress release vocalizations in *Rana pipiens* (Diakow et al., 1978). Female *X. laevis* may emit unreceptive vocalizations upon male detection (Kelley 1982), whereas *R. pipiens* females only vocalize after being clasped (Diakow et al., 1978).

While amplexus modifies steroid levels in gray treefrogs, other cues must be responsible for the steroid surges themselves that are observed on breeding nights. Levels of estradiol, progesterone and testosterone are all greatest in pre-amplectant

females, relative to amplectant or non-breeding females (Gordon and Hellman, Chapter 2). Because these reproductive steroids are all elevated prior to the arrival of an amplectant male, our evidence suggests that amplexus is only acting to slow the decline of testosterone and estradiol - it does not cause the initial elevation of these steroids. Furthermore, because estradiol levels did not increase with time in amplexus, it is likely that either the onset of amplexus or a cue correlated with the onset of amplexus is responsible for the elevation of this steroid. Females that took longer to oviposit did have greater testosterone levels, so time in amplexus does appear to influence plasma testosterone.

Oviposition behavior

Almost all females in our study oviposited, regardless of the presence or absence of acoustic stimuli or an amplectant male. Because we captured frogs moving toward the chorus, females are apparently coming to breeding ponds having already committed to oviposition. Females of the barking treefrog, *Hyla gratiosa*, approach breeding ponds even when all males are removed prior to chorusing, which suggests that these treefrogs do not require acoustic stimulation to "decide" when reproduction should occur (Murphy 2003). We could only minimize, and not eliminate acoustic exposure of females to male signals prior to arrival at the breeding pond in our study. However, we did control for acoustic exposure in the period that would have encompassed pond arrival and beyond, and we noted no effect of these signals on oviposition probability. Consequently, though male signals may facilitate other reproductive events such as ovulation or phonotaxis, it does not appear that they are necessary for oviposition in gray treefrogs. Amplexus (or a

cue correlated with it) appears to be a cue necessary for oviposition in other treefrogs, however; barking treefrogs will not oviposit without amplectant males (Scarlata and Murphy 2003).

We found that the timing of oviposition is influenced by conspecific signals, though not in the expected direction. Darling (1938) initially proposed that social stimulation would hasten oviposition, and several studies in a variety of taxa (including frogs) have supported this hypothesis or suggested that conspecific signals should hasten reproductive events in general (McComb, 1987; Cheng, 1992; Lea *et al.*, 2001; Kroodsma 1976; Waas et al., 2005). Our findings do not support Darling's (1938) hypothesis and show that in female gray treefrogs, oviposition is delayed in the presence of male calls. Because oviposition occurred regardless of whether a male was present for amplexus, females are not delaying oviposition until a male arrives. This delay could be a side effect of estradiol elevation. If a decline in estradiol is a signal for oviposition, then an increase in estradiol stimulated by male calls might counteract this signal somewhat. Because even the call-delayed oviposition still occurred before sunrise, there may not be a significant negative effect of delaying oviposition by the hour or so noted here. Furthermore, the benefits of promoting receptivity, discussed above, may outweigh any cost of time. Further work will be needed to clarify the role of estradiol in the timing of oviposition.

Conclusion

Here we have shown that both auditory and amplexus-related social signals influence two steroids associated with reproductive behavior. Our data support the

hypothesis that social stimulation slows the decline of reproductive hormones after breeding events, and we provide the first evidence that amplectant males influence these hormones in female frogs. Our data do not support the hypothesis that social stimulation hastens reproduction, since we have shown oviposition took longer in the presence of acoustic stimulation.

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FIGURES

Figure 1: Oscillograms (top) and spectrograms (bottom) of pink noise (A) and call and chorus (B) stimuli recorded at cage front.

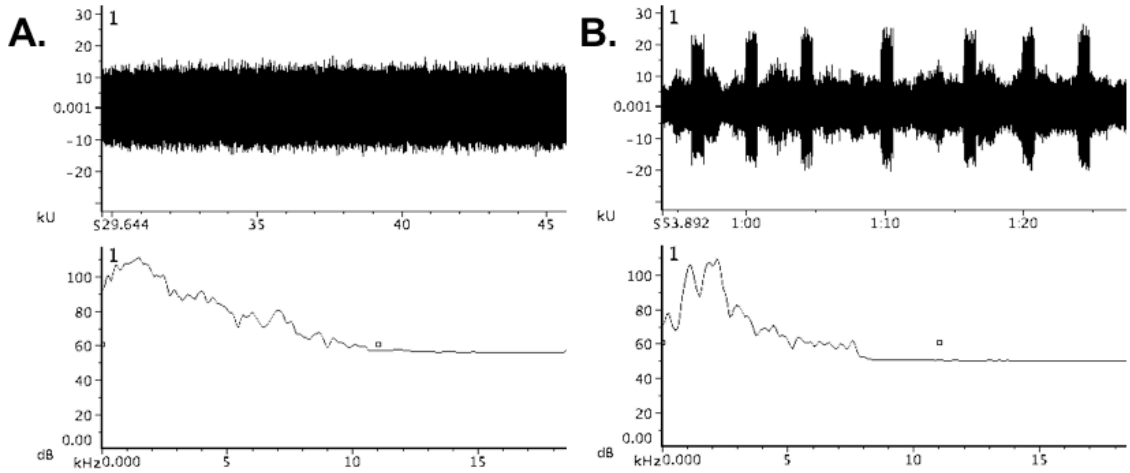


Figure 2: Percent of females ovipositing after exposure to one of three acoustic treatments.

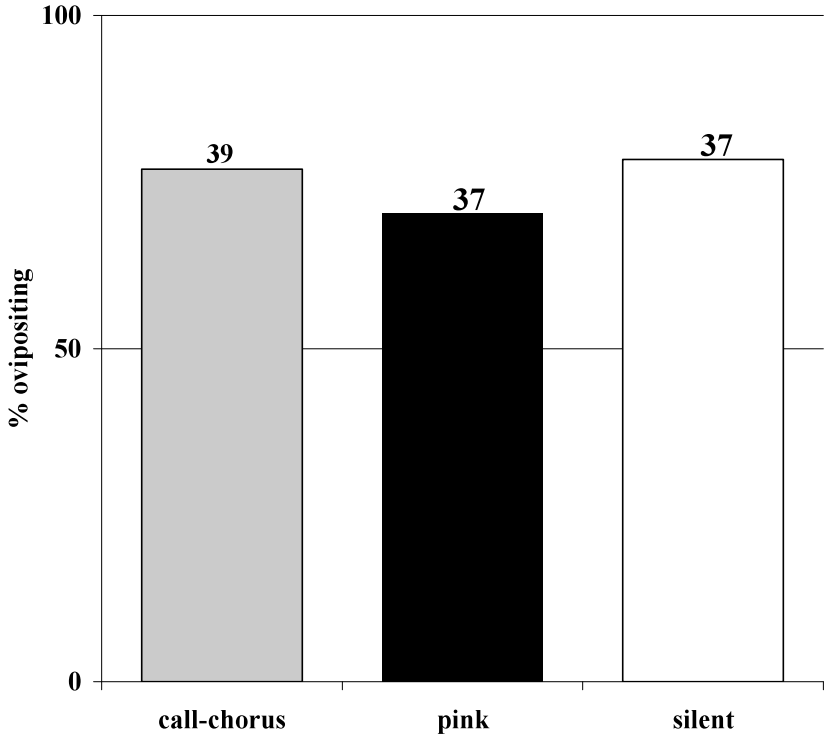


Figure 3: Mean (±S.E.) time till oviposition of amplexed and unamplexed females exposed to three acoustic treatments.

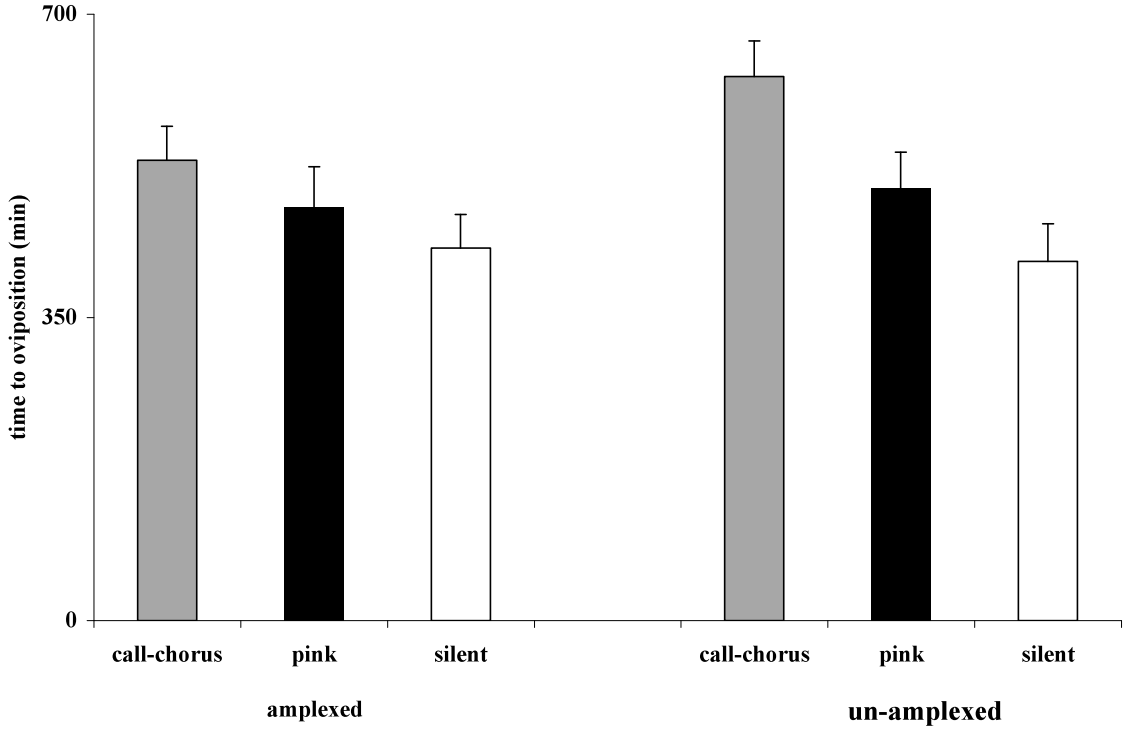


Figure 4: Relationship between plasma testosterone (A), estradiol (B), and progesterone (C) and the time it took females to oviposit.

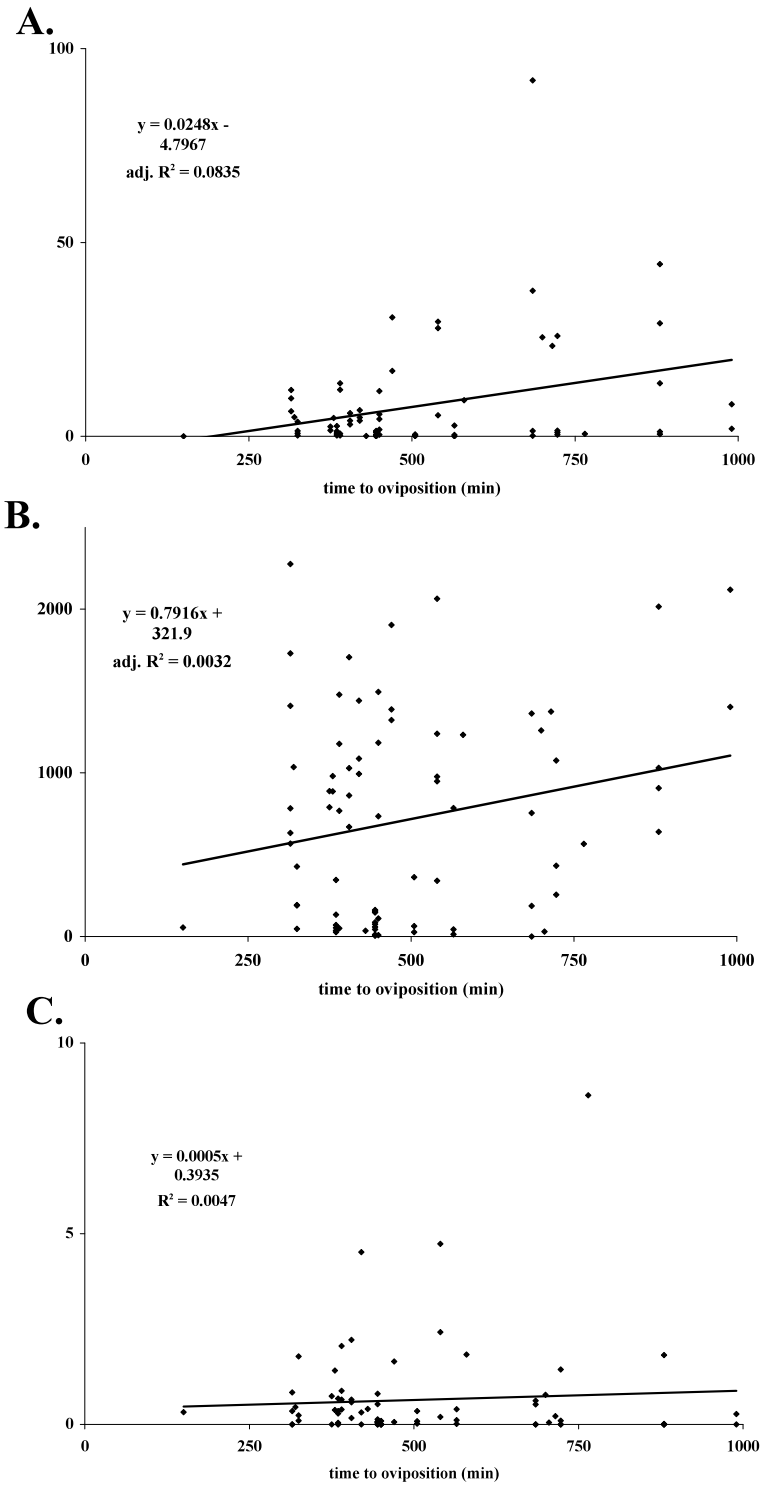


Figure 5: Comparison of plasma steroid levels after exposure to acoustic stimuli and prior to amplexus and oviposition. * denotes significant at p=0.0608. Values are means±S.E.

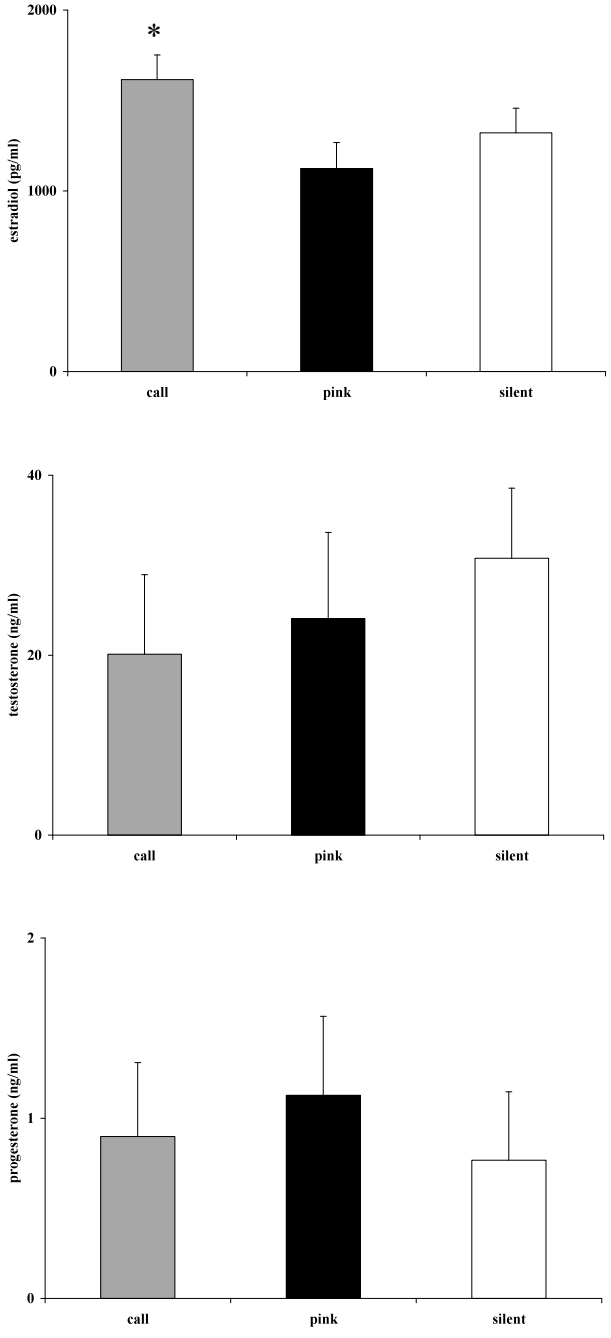
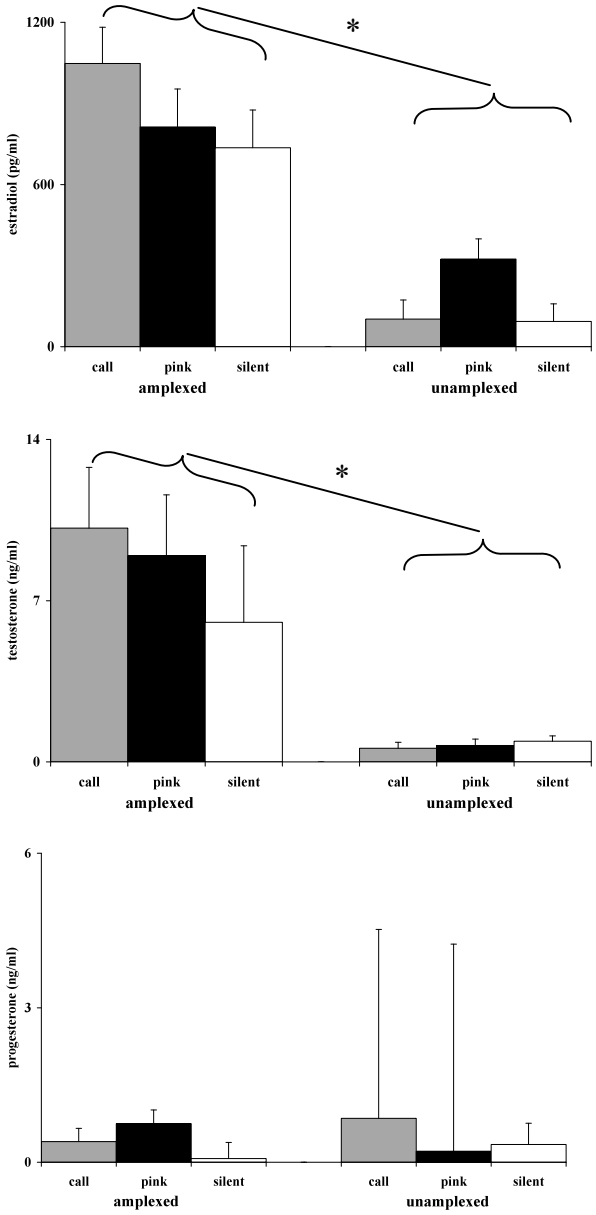


Figure 6: Comparison of post-oviposition steroid levels in amplexed and unamplexed females exposed to different acoustic treatments. * denotes $p<0.0001$). Values shown are means±S.E.



Chapter 4

Exposure to acoustic signals does not influence reproductive steroids and behaviors in female treefrogs over prolonged periods.

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ABSTRACT: Steroid hormones regulate reproduction and influence behaviors, but how hormones respond to exogenous cues is poorly understood. I explored how the presence of acoustic social signals influences the hormones and behaviors that signal female reproductive events in the gray treefrog, *Hyla versicolor*, over the time scale of an entire breeding season. I investigated the hypothesis that exposure to male advertisement calls increases the likelihood of reproductive events over prolonged periods. I tested three specific predictions about the effects of such exposure: (1) that ovarian development would be accelerated and the period between peak plasma levels in steroids associated with reproduction would be shortened; 2) that phonotaxis behavior would be more likely; and 3) that amplexus behavior would be more likely. Females were exposed to acoustic stimuli either for the duration of a breeding season or the period between winter brummation and first reproduction. Female treefrogs that heard conspecific male signals exhibited no increase in the number of biologically significant plasma estradiol or progesterone peaks and were not more likely to exhibit phonotaxis or amplexus than were females that heard pink noise or silence. My data do provide some support for the

hypothesis that acoustic stimulation promotes ovarian development in some situations; however, further work will be required to determine the exact nature of this relationship. My findings show that in female gray treefrogs, hormonal, ovarian and behavioral signals of reproduction are generally not promoted by prolonged exposure to conspecific signals in biologically relevant ways.

INTRODUCTION

In many animals hormones have been shown to regulate reproductive cycles and to influence reproductive behaviors (e.g. Vandenbergh and Drickamer 1974, Crews and Moore, 1986). While previous workers have shown correlations between hormone levels and reproductive behaviors across years or breeding seasons (e.g. Wingfield *et al.* 1997; Kim *et al.*, 1998), the cues triggering these changes remain poorly understood.

The exogenous cues that trigger female reproduction are varied. Such cues may be environmental (e.g. day length, humidity or temperature), physiological (e.g. fat stores), or behavioral (e.g. physical mate contact or sexual advertisement signals) (Herman, 1992). Conspecific acoustic signals can act as a cue regulating reproduction. Male roaring can advance the date of oestrus in female red deer, *Cervus elaphus* (McComb, 1987), and ring doves, *Streptopelia risoria*, that hear their own vocal signals show increased ovarian development (Cheng, 1992).

There is mounting evidence that vocal signals could also act to influence reproductive states in female frogs. Female Mallorcan midwife toads (*Alytes muletensis*) maintain reproductive condition and do not reabsorb their eggs in the presence of male

calls (Lea *et al.*, 2001). Because oocyte maturation and re-absorption is controlled by reproductive steroids (Lin and Schuetz 1983 and 1985), the findings of Lea *et al.* (2001) strongly suggest that calls are influencing hormonal levels in this species. Estradiol levels in in captive female tungara frogs (Physalaemus pustulosus), increased in response to male signals (Lynch and Wilczynski 2006) in a manner similar to that observed in wild females (Lynch and Wilczynski 2005). This estradiol increase may promote anuran phonotaxis (Gordon and Gerhardt in press; Chakraborty and Burmeister, in press), and it likely plays a role in other reproductive events as well, such as vitellogenesis and follicular growth (Bruscalupi et al., 1998; Pierantoni et al., 1987).

Female gray treefrogs have opportunities to hear males calling throughout the non-brummation period, though calling is much more frequent during the breeding season. Males begin calling sporadically shortly after emerging from brummation. These males are distributed away from the breeding ponds, and move toward the pond as the breeding season approaches. Males can be heard calling for periods of days to weeks before the first females arrive at the breeding pond. Consequently, because females emerge from brummation at similar times as males (Johnson et al., 2007, NMG pers. obs.), there is a period (9.4±1.8 days, over 5 years at our study site) during which females can hear male calls prior to the first observed reproduction. Levels of both plasma estradiol and progesterone are at their annual peak on breeding nights (Gordon and Gerhardt, chapter 2), so increased levels of these hormones almost certainly signal reproductive events.

Steroid hormones regulate reproduction and influence behaviors, but how hormones respond to exogenous cues is poorly understood. Previously I have shown that

male calls influence estradiol levels and oviposition behavior in gray treefrogs over the time scale of a single breeding night (Gordon and Gerhardt, chapter 3). Here I test three predictions stemming from the general hypothesis that male advertisement calls increase the likelihood of female reproductive events over a more prolonged period. I predicted that exposure to male calls relative to control stimuli will: 1) hasten ovarian development and shorten the period between peak plasma levels in steroids associated with reproduction; 2) increase the likelihood of phonotaxis behavior; and 3) increase the likelihood of amplexus behavior.

METHODS

All procedures outlined in this study were approved by the University of Missouri Animal Care and Use Committee protocol #1910. Animals were collected under Missouri Department of Conservation Wildlife collector's permits #12343 and #13232. Gray treefrog, *Hyla versicolor*, amplexed pairs were initially collected by hand at breeding chorus in the Thomas Baskett Wildlife Conservation Area near Ashland, MO, USA during the month of April, 2004 - 2006. Pairs were brought to facilities at the University of Missouri and left overnight to oviposit, males and oviposited eggs were returned to the breeding pond, and females were housed individually for subsequent experiments.

Experiment 1: monitoring seasonal changes

All females were individually housed in acoustically transparent cages with a water bowl, perch site and damp sphagnum moss. Cages were maintained in windowless

rooms under fluorescent light at 20-24 ^{o}C with a 14L/10D light cycle. Females were fed crickets ad libitum, and water was changed at least weekly. Females were then exposed to one of three stimuli broadcast from portable CD players (Audiovox DM8220B, Hauppauge, New York, USA), amplifiers and speakers (Radio Shack SA-10, Fort Worth, Texas, USA). The sound pressure level (SPL re 20 μPa, C-weighting, fast root-mean-square) of the stimuli was equalized at the cage front with a Radio Shack 33-2055 sound level meter. Anechoic foam was placed behind cages on the side opposite the speakers to minimize sound reflection. All cages were placed in acoustically isolated rooms, the location of cages within a room was changed weekly and cages were rotated between rooms three times during the course of the experiment to minimize location effects. Females were assigned to treatments randomly, and an individual female heard the same acoustic stimuli for the duration of the experiment regardless of her location (i.e. acoustic treatments followed individual frog-cages to different locations).

I used five acoustic treatments, two in 2005 and three in 2006. In 2005 the acoustic treatments were pink noise, and a combination of conspecific call and chorus noise (see Fig. 1 for oscillograms and spectrograms of these stimuli). In 2006 I used the call- and chorus-noise stimuli separately, and silent, unpowered speakers as the third treatment. All stimuli were broadcast night and day in a continuous loop for the duration of the experiment without breaks, except when cages were being rotated between rooms, a process that took approximately 2 hrs. Pink noise was generated using Cool Edit (Syntrillium Co, Phoenix, AZ, USA). Pink noise was used rather than white noise or random tones because the pressure spectrum level of pink noise declines logarithmically with increasing frequency, generally mimicking the sensitivity of vertebrate auditory

neurons, and hence all auditory receptors would be equally stimulated. Frogs exposed to the silent treatment were tested with an unpowered speaker where background sounds (noise from the air conditioning fans were most prominent), were approximately 40 dB SPL or less and shielded with anechoic foam. The call and chorus stimulus consisted of a synthetic "average" male call 837 ms (18 pulses) long with a pulse rate of 20 pulses/s; the spectrum consisted of two components of 1.1 and 2.2 kHz, with the amplitude of the low-frequency component 6 dB less than that of the high-frequency component. The call period was 4 s. In two-speaker tests, females responded as often to this standard synthetic call as they did to pre-recorded exemplars (Gerhardt 1978). This synthetic call was added to a recording of chorus noise from the pond where females were collected, and adjusted to be 6 dB greater than the chorus noise for 2005, in 2006 the call and chorus noise were broadcast as separate stimuli. The call was synthesized using custom designed software (by J. Schwartz) and modified using Cool Edit (Syntrillium Co, Phoenix, AZ, USA). The call and chorus noise stimuli was broadcast at 83 dB SPL at the cage front during the synthetic call portion of the stimuli, which is the approximate SPL of male calls from our study population at 1 m. The pink noise stimulus was set to 79 dB SPL. I used different sound pressure levels for the pink noise and conspecific calls in an attempt to approximately equalize the total energy over time of the two stimuli, since pink noise has a constant SPL over time, whereas the call and chorus noise stimuli has a variable SPL over time. I used a Larson Davis SPL meter (800B, Provo, Utah, USA) to measure and calibrate stimuli over a longer time window (slow RMS setting: one second) and then measured the calibrated signals with the Radio Shack SPL meter to determine the experimental fast RMS setting.

To assess how my treatments might be influencing reproductive steroid levels a sample of blood (~100 µl) was collected from a subset of females weekly. Individual females were sampled bi-weekly, such that half the frogs exposed to each treatment were sampled each week. Frogs were sampled for 10 weeks, the approximate duration of the breeding season in Missouri. Samples were taken from each frog via cardiac puncture. Blood samples were stored up to 24 hr at 2-8 °C and then centrifuged to separate and remove the plasma. Plasma was then stored at -20 °C until assayed.

Hormonal analyses were done with commercial radioimmunoassay kits (progesterone: Coat-a-Count TKPG-2, Siemens, Los Angeles, CA; estradiol: ImmuChem 07-138102, MP Biomedicals, Orangeburg, NY). All samples were run in duplicate. Samples were diluted to 10 µl sample in 90 µl zero standard prior to assay. Kits were validated using serial dilution of a single pooled sample mixed from several frogs. Curves generated from these serial dilutions were parallel to the standard curves generated for each hormone (data not shown). Mean intraassay coefficients of variation for progesterone were 17.2% and for estradiol were 16.0% (all based on 5 standards run with each assay). Interassay coefficients of variation were 8.7% for four progesterone assays and 24.2% for thirteen estradiol assays. The minimum detection limit was 0.1 ng/ml for the progesterone assay and 10 pg/ml for estradiol. I was not able to obtain blood samples from all animals, and some blood samples were too small to allow for assaying both steroids, consequently, sample sizes are not always equivalent.

All females were weighed weekly (after blood sampling, if applicable) and the state of their ovaries was assessed. I assessed the development of ovaries visually by holding frogs up to a microscope flex light and examining the ovaries through the body

wall. Ovaries were scored as being in one of four stages, representing increased degrees of development after oviposition (Fig. 2): stage 1 - no ovaries visible; stage II - small ovaries visible, but individual follicles are not discernable; stage III - ovaries visible and some follicles are large enough to be individually identified; stage IV - eggs have ovulated and a jelly coat is visible around individual eggs. All scoring was done by the same individual using figure 2 as a guide, with corroboration by an independent observer. Occasionally gut contents obscured view of the ovaries preventing a stage assignment. In these situations I assigned a stage by averaging the previous and next week's stage assignments. Twenty of 737 observations in 2006 and 46 of 441 in 2005 were assigned in this manner.

Experiment 2: influence of acoustic stimuli on post-brummation phonotaxis

Females brummated in their individual cages at 2-5 °C for 3 months (January-March), and the light cycle was reduced to 8L/16D. At the conclusion of brummation, cages were warmed to 20 °C and feeding was resumed. All females were then exposed to one of the three acoustic stimuli outlined above (call and chorus, pink noise or silence). Stimuli were broadcast continuously for two weeks.

I tested for phonotaxis behavior using a single speaker design in a 2 m-diameter circular arena within a semi-anechoic chamber at 20±1°C. The arena was bordered by a wire mesh fence 1 m high and covered with black, acoustically transparent fabric. A single Analog-Digital-Systems 200 speaker was placed just outside the arena flush against the mesh. The speaker broadcast a single male call (the synthetic call described above, without the addition of the background chorus noise). For each test, a female was

placed in a small hardware cloth cage in the center of the arena. The SPL of this stimulus was adjusted to 85 dB SPL at this release point with a Larsen-Davis 800B sound level meter. Females were released by remotely removing the top of the cage after the stimuli was broadcast at least three times.

During testing, frogs were observed with a remote camera and infrared illumination. A response was tabulated when a female moved to within 10 cm of the speaker after showing phonotactic orientation movements, such as head and body scanning (Rheinlaender et al., 1979). I also recorded the time to make a choice. A "no response" was recorded when the female failed to show phonotactic behavior within 15 min of release; some females remained in the release cage and others wandered around randomly in the chamber without showing phonotactic orientation movements.

Experiment 3: influence of acoustic stimuli on post-brummation amplexus

Females were brummated and exposed to stimuli as in experiment 2; in this experiment, however, there was no silent treatment because of an insufficient number of females. Females used in this experiment had been toe clipped for individual identification prior to brummation.

I conducted this experiment on a single night in a large (approx. 4 m diameter) artificial pond inside a greenhouse on the University of Missouri campus. The pond was constructed of a plastic liner approximately 10 cm deep and enclosed above by a mesh and wood frame. PVC pipe and wooden dowels were placed on stands along the periphery of the pond to act as perch sites.

One day prior to testing I collected 25 calling males from our breeding pond and released them in the artificial pond. Males called vigorously after capture and did not appear to be affected by the change in location. On the following morning I placed all the females in their cages on the floor of the artificial pond in an alternating circular pattern such that females that had heard the pink noise treatment were next to females that heard the call-chorus treatment. All cages were then opened such that females could climb out and move about the artificial pond, and the frogs were left undisturbed till sunset. Male treefrogs do occasionally call sporadically during daylight hours, though I did not hear any of these males calling until approximately an hour prior to sunset.

After sunset and the commencement of chorusing, I searched the pond every half hour for the presence of amplectant pairs. I captured any observed pairs, noted the time of capture and placed them in individual containers with water for oviposition (all females captured in this manner oviposited). After oviposition I identified the individual females using her toe clips. I continued searching the pond in this manner until 2 hrs after the cessation of chorusing. The following morning I checked the pond for any signs of oviposition (no eggs were found) and all remaining frogs were removed.

Statistical analyses

I tested for influence of acoustic treatment on ovarian stages and plasma hormone levels over time using repeated measures analyses. Ovarian stages were analyzed with a repeated measures MANOVA in JMP. Insufficient plasma sample volumes meant that I was unable to obtain hormone levels for every female at every time interval.

Consequently I used a mixed model (PROC MIXED in SAS) to perform repeated measures analysis on the hormonal data.

Because the repeated measures analyses could be confounded by a preponderance of near baseline hormone levels, I re-analyzed these data using only peak hormone values, and tested the null hypothesis that the number of peak values was the same between treatment groups. I obtained peak hormone levels by excluding all values within one standard deviation of the mean value for non-breeding females near the study pond (values less than 125 pg/ml for estradiol and less than 1.68 ng/ml for progesterone were excluded, both based on Gordon and Hellman, chapter 2). I then counted the number of remaining values (= number of hormonal peaks) per female and analyzed whether the mean number of peaks differed between the acoustic treatments using one-way ANOVA.

I tested whether acoustic treatment influenced the proportions of females that exhibited phonotaxis or were amplexed with Chi-square contingency analyses. The influence of acoustic treatment on the time it took females to exhibit phonotaxis or amplexus was analyzed with one-way ANOVA.

RESULTS

Experiment 1: monitoring seasonal changes

There was little influence of acoustic treatments on hormone levels. Using repeated measures analyses, the only significant effect of acoustic treatment was on progesterone levels in 2005. Females exposed to call and chorus noise had significantly greater plasma progesterone on average over the course of the season in 2005 compared

to females exposed to pink noise ($F_{1,125}$=3.45, p=0.0655) (Fig. 3A). Progesterone levels were not influenced by acoustic stimuli in 2006 ($F_{2,138}$=0.92, p=0.4025) (Fig. 3B), and estradiol levels were not influenced by acoustic stimuli in either year (2005: $F_{1,144}$=0.02, p=0.8782; 2006: $F_{2,274}$=0.28, p=0.7538) (Fig. 4AB).

Acoustic treatment did not influence the number of hormonal peaks above the levels typical of non-breeding females. The number of estradiol peaks was not influenced by acoustic treatment in either year (2005: $F_{1,47}$=1.7738, p=0.1893; 2006: $F_{2,65}$=0.2590, p=0.7726) (Fig. 5AB). Only one female had a progesterone level greater than the cutoff value over the two years of this study (this female was exposed to the silent treatment), so progesterone peaks were not analyzed.

The influence of acoustic treatment on ovarian stage varied across the two years of this study. There was no significant difference in ovarian stages between females exposed to pink noise or call and chorus stimuli in 2005 ($F_{1,47}$=1.0935, p=0.3010). However, ovarian stage was significantly less in females exposed to the silent treatment in 2006 compared to either of the conspecific stimuli ($F_{2,70}$=4.0250, p=0.0221) (Fig. 6AB).

The number of weeks required for females to reach ovarian stage III (= the first observation of individual follicles) was influenced by acoustic treatment in 2005 but not 2006. Females exposed to pink noise took longer to reach stage III than females exposed to call and chorus stimuli ($F_{1,47}$=4.0016, p=0.0513). There was no influence of acoustic treatment on ovarian development in 2006 ($F_{2,70}$=0.2458, p=0.7827) (Fig. 7AB).

Experiment 2: influence of acoustic stimuli on post-brummation phonotaxis

There was no significant influence of acoustic treatment on the number of females exhibiting phonotaxis post-brummation (n=60, χ^2=0.489, p=0.7830) (Fig. 8A). Most females (41 of 60) did not exhibit phonotaxis behavior at all and merely wandered about the test arena. For females that did respond, acoustic treatment did significantly influence the time it took females to respond ($F_{2,16}$=3.7216, p=0.0471) (Fig. 8B): females that had previously heard call and chorus noise took significantly longer to respond than did females that had heard pink noise ($F_{1,10}$=10.7718, p=0.0083). There was no significant difference in response time for females that heard call and chorus noise previously compared to frogs in the silent-treatment group ($F_{1,12}$=0.5574, p=0.4697).

Experiment 3: influence of acoustic stimuli on post-brummation amplexus

I detected no significant effect of acoustic stimuli on post brummation amplexus. Females that heard call and chorus stimuli (n=9) prior to the night males were first encountered were not more likely to be found in amplexus compared to females that had previously heard pink noise (n=8) (χ^2=0.084, p=0.7713) (Fig. 9A). The time it took females to reach these males was also not significantly different between the two acoustic treatments ($F_{1,5}$=0.1342, p=0.7291) (Fig. 9B).

DISCUSSION

Previously we have shown that male advertisement calls influence estradiol levels and oviposition behavior in gray treefrogs over the time scale of a single breeding night

(Gordon and Gerhardt, chapter 3). Here I show that male calls do not increase the likelihood of female reproductive events in a biologically relevant way over more prolonged periods. Exposure to male calls relative to control stimuli did not: 1) hasten ovarian development or the period between peak plasma levels in steroids associated with reproduction; 2) increase the likelihood of phonotaxis behavior; or 3) increase the likelihood of amplexus behavior.

In the wild, male treefrogs can be heard constantly on breeding nights and sporadically throughout the remained of the day, but periods of relative silence are also common. My stimuli were broadcast continuously, so I cannot rule out the possibility that constant stimulation was perceived negatively by female treefrogs. Frogs exposed to the silent treatment did not exhibit substantial differences from frogs exposed to other acoustic stimuli in the reproductive parameters measured, suggesting that either constant stimulation was not perceived negatively, or that it was perceived equally to constant stimulation. Furthermore, I cannot exclude the possibility that females habituated to the tested stimuli, and the lack of significant effects was a product of habituation.

Experiment 1: monitoring seasonal changes

I have shown that what a female hears across a breeding season generally did not influence her plasma progesterone or estradiol levels over prolonged periods. Progesterone levels were marginally greater in females that heard call and chorus noise compared to females that heard pink noise; however, none of the recorded progesterone levels in this study approached levels typical of breeding gray treefrogs (Gordon and Hellman, chapter 2). In fact, only one blood sample from this entire study had a

progesterone level greater than that typical for non-breeding season females (Gordon and Hellman, chapter 2). This observation suggests that no females in this study were stimulated enough to sufficiently increase hormones in anticipation of reproduction. There was no difference in progesterone levels in females that heard synthetic calls, natural chorus noise, or silence. Consequently, it may be more likely that pink noise acted to suppress progesterone rather than the call and chorus stimuli increasing progesterone above baseline levels. Most females did exhibit peaks in estradiol levels that approached or exceeded levels found in naturally breeding females; however, acoustic treatment did not influence the number of these peaks.

Wilczynski and colleagues (2005) hypothesized that gonadal hormones in frogs would decline more slowly across a breeding season in the presence of conspecific acoustic stimulation. My data do not support this hypothesis with regard to progesterone or estradiol in gray treefrogs. Short-term elevation of estradiol in response to conspecific social stimulation has been noted before in female frogs (Lynch and Wilczynski 2006; Gordon and Gerhardt chapter 3). My data provide the first test of this hypothesis over an entire breeding season.

My study provides further support for the hypothesis that elevation of estradiol in response to conspecific signals is a transient event in gray treefrogs. It has been suggested that using acoustic signals to elevate steroids could be a mechanism for promoting the maintenance of reproductive condition for future breeding events (Wilczynski et al., 2005; Lynch and Wilczynski 2006). If acoustic signals served to promote maintenance of reproductive condition through steroid elevation, then I would expect that exposure to such signals would reduce the decline or rate of decline in steroid levels after

reproductive events. Because hormone levels in all gray treefrog females declined to similar levels after oviposition, regardless of acoustic experience, I suggest that acoustic stimulation is not promoting maintenance of reproduction beyond breeding events, at least through hormonal elevation.

It is still possible that acoustic signals influence the likelihood of future reproduction via a non-hormonal mechanism, or that temporary hormonal changes (e.g. Lynch and Wilczynski 2006; Gordon and Gerhardt chapter 3) trigger some other physiological event that affects future reproduction, but these hypotheses will require further testing. One possibility is that temporary hormonal surges could promote ovarian development. In addition to hypothesizing that hormone levels would decline more slowly, Wilczynski and colleagues (2005) also hypothesized that gonadal state in frogs would decline more slowly across a breeding season in the presence of conspecific acoustic stimulation. Here I have shown that acoustic stimulation did influence ovarian development over the course of a breeding season. Females that heard calls or chorus noise had greater ovarian development in the last few weeks of the breeding season than did females in the silent-treatment group. There was no detectable difference in ovarian development of females that heard call and chorus noise compared to those that heard pink noise. The design of my study precludes statistical comparison of the pink noise and silent treatments because they were conducted in different years for logistical reasons. Thus, I cannot exclude the possibility that any acoustic stimulation would be sufficient to maintain ovarian development. Furthermore, individual follicles were observed significantly earlier in females that heard call and chorus stimuli relative to females that

heard pink noise. However, for the reasons previously noted I cannot discern if this was due to suppressive effects of pink noise or stimulation due to conspecific signals.

Experiments 2 and 3: influence of acoustic stimuli on post-brummation phonotaxis and amplexus

In the wild, females are active for periods of days to weeks between emerging from brummation and the first breeding activity of the season. Males call sporadically during this period, so females are exposed to some conspecific acoustic stimulation. My data suggest that this exposure to male advertisement calls is not influencing the probability of females exhibiting phonotaxis or amplexus. Sample sizes were very low for the test comparing the likelihood of amplexus, however, taken together with my other results it seems unlikely that increased power would change the overall conclusion of no effect.

While I did not find an influence of prior acoustic stimulation on the time it took females to pair with males, there was a reduction in phonotaxis time for females that had previously heard pink noise relative to call and chorus noise. There was no difference in response time for females exposed to call and chorus noise compared to silence, so my data suggest that pink noise exposure hastened phonotaxis. This result is difficult to interpret - it is possible that pink noise was perceived negatively and consequently generated a more positive response when it was removed. In fact, it could also be possible that both stimuli are perceived negatively when presented constantly, and the call-chorus stimuli was only less so. Regardless of one's interpretation, my results do not

provide support for the hypothesis that extensive (prolonged) exposure to conspecific signals prior to breeding nights is promoting reproduction.

Conclusion

Darling (1938) initially proposed that social stimulation would hasten oviposition, and several studies in a variety of taxa have supported this or have suggested that conspecific signals should hasten reproductive events in general (McComb, 1987; Cheng, 1992; Lea *et al.*, 2001; Kroodsma 1976; Waas et al., 2005; Lynch and Wilczynski 2006). My findings do not support Darling's (1938) hypothesis and show that in female gray treefrogs, hormonal, ovarian and behavioral signals of reproduction are generally not promoted by prolonged exposure to conspecific signals in biologically relevant ways. My data do provide some support for the hypothesis that acoustic stimulation influences ovarian development in some situations; however, further work will be required to determine the exact nature of this relationship.

The cues that promote reproduction in frogs are poorly understood. My study suggests that acoustic signals are not being used as a cue in gray treefrogs. Warm spring days and rainfall are associated with the beginning of the breeding season, but at least some females in a population can be found breeding on almost every night thereafter for a period of several months. Therefore climatic variables may be correlated with population level breeding activity but may not be reliable for predicting individual level variation. Individual reproductive events may consequently be more dependent on the foraging success of the female (Ritke and Lessman 1994) than on seasonal/

environmental cues (Alexander and Bellerby 1938), or on exposure to conspecific signals (this study).

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FIGURES

Figure 1: Oscillograms (top) and spectrograms (bottom) of pink noise (A) and call and chorus (B) stimuli recorded at cage front.

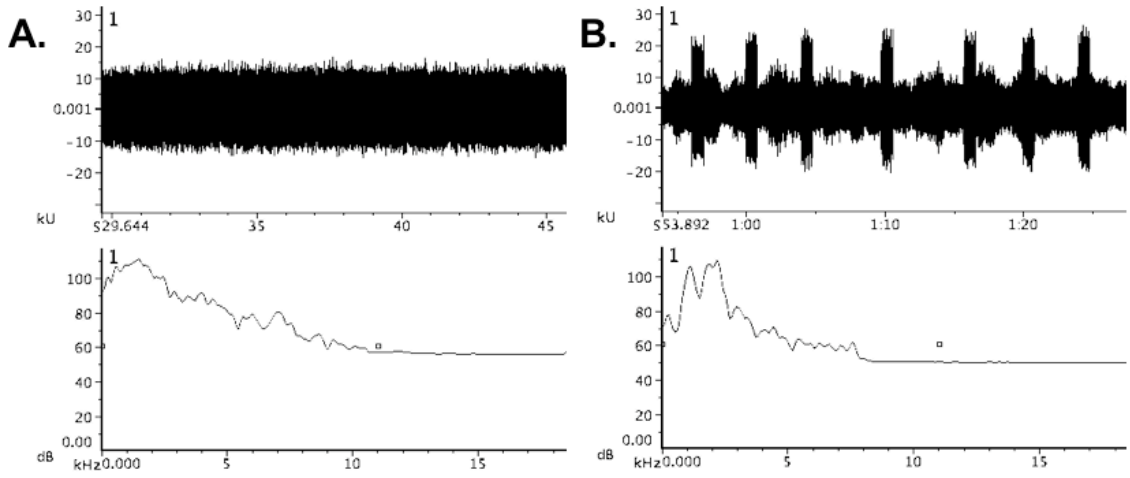


Figure 2: Ovaries were examined by shining a focused light through females in a dim room. Ovaries were scored as being in one of four stages, representing increased degrees of development after oviposition: stage I - no ovaries visible; stage II - small ovaries visible, but individual follicles are not discernable; stage III - ovaries visible and some follicles are large enough to be individually identified; stage IV - eggs have ovulated into body cavity and jelly coat is visible. All frogs are shown in ventral view with the anterior oriented to the right and hind legs extended to the left.

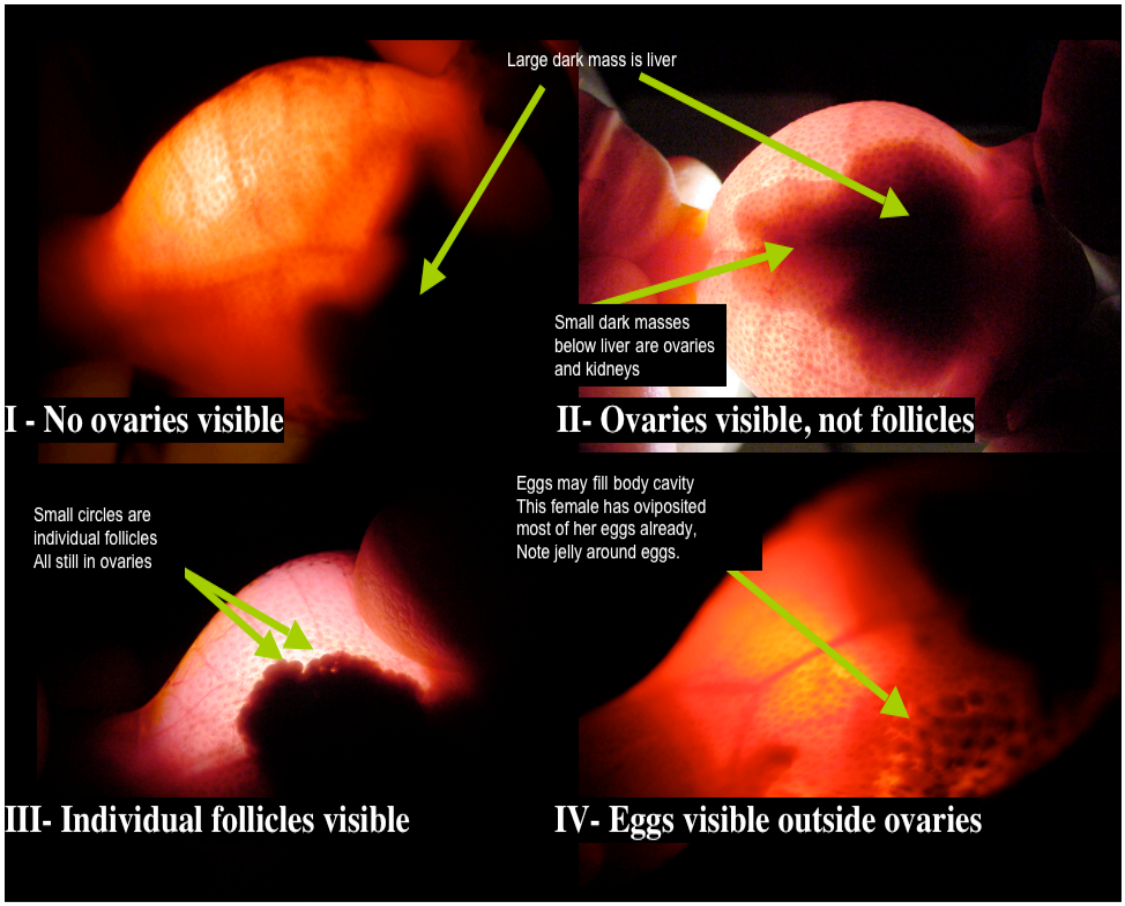


Figure 3: Mean ±S.E. levels of progesterone measured across two breeding seasons in females exposed to different acoustic treatments. (A) Females exposed to call and chorus noise had greater plasma progesterone on average over the course of the season in 2005 compared to females exposed to pink noise. (B) Progesterone levels were not influenced by acoustic stimuli in 2006.

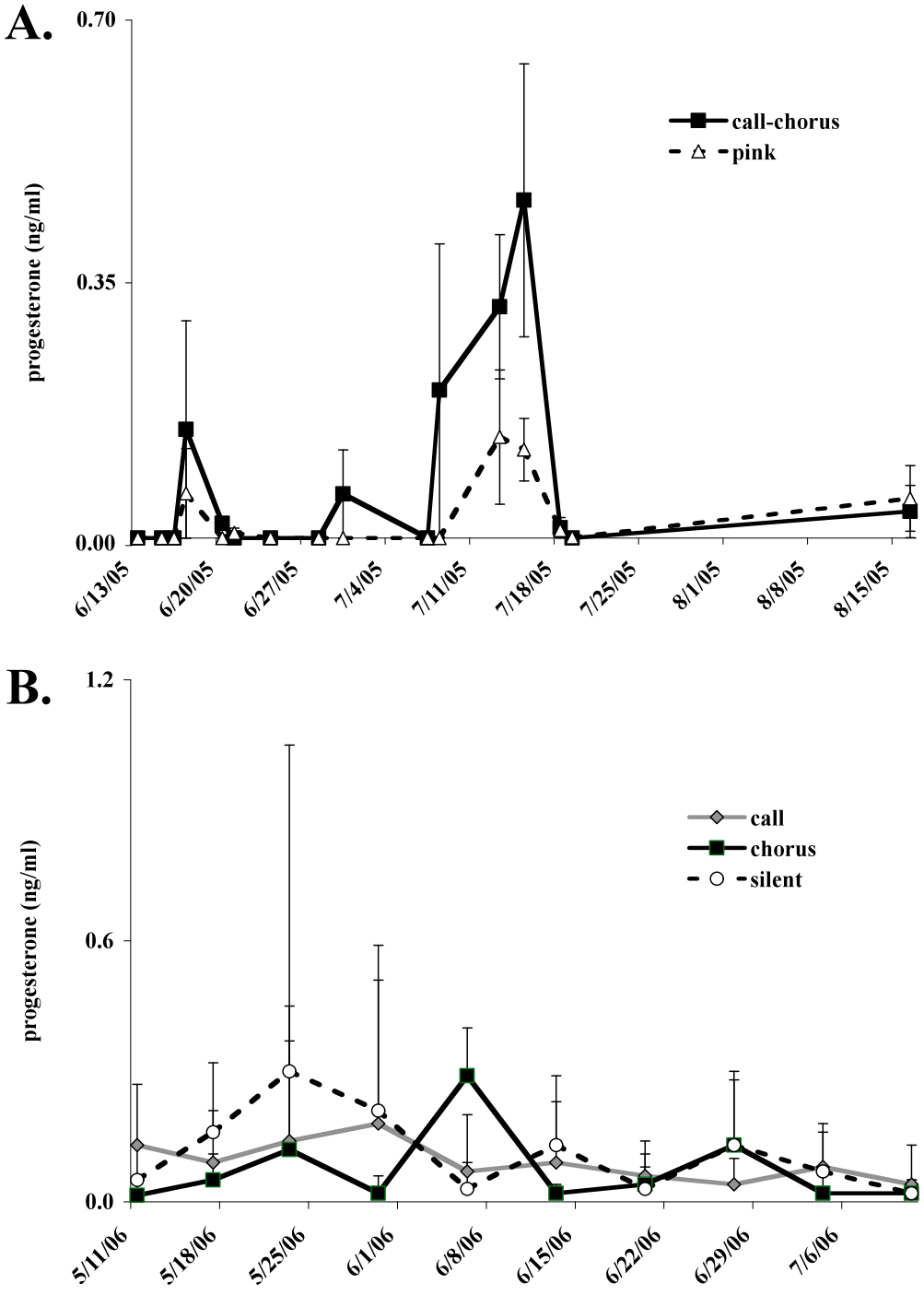


Figure 4: Mean ±S.E. levels of estradiol measured across two breeding seasons in females exposed to different acoustic treatments. Estradiol levels were not influenced by acoustic stimuli in (**A**) 2005: p=0.8782, or (**B**) 2006: p=0.7538.

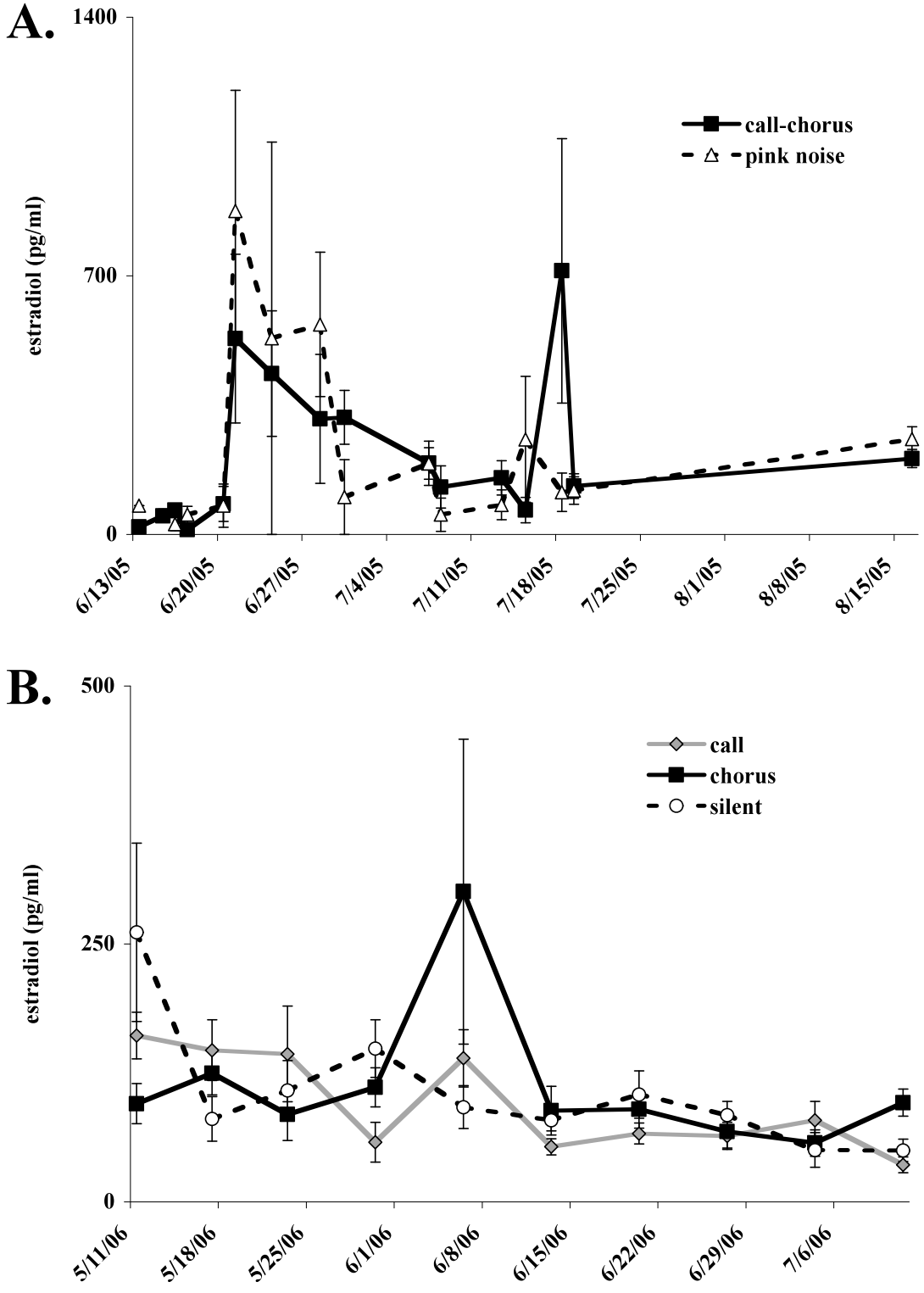


Figure 5: Acoustic treatment did not influence the number of hormonal peaks above levels typical of non-breeding females. (A) 2005: p=0.1893, (B) 2006: p=0.7726. Values are means ±S.E. Numbers above bars are sample sizes.

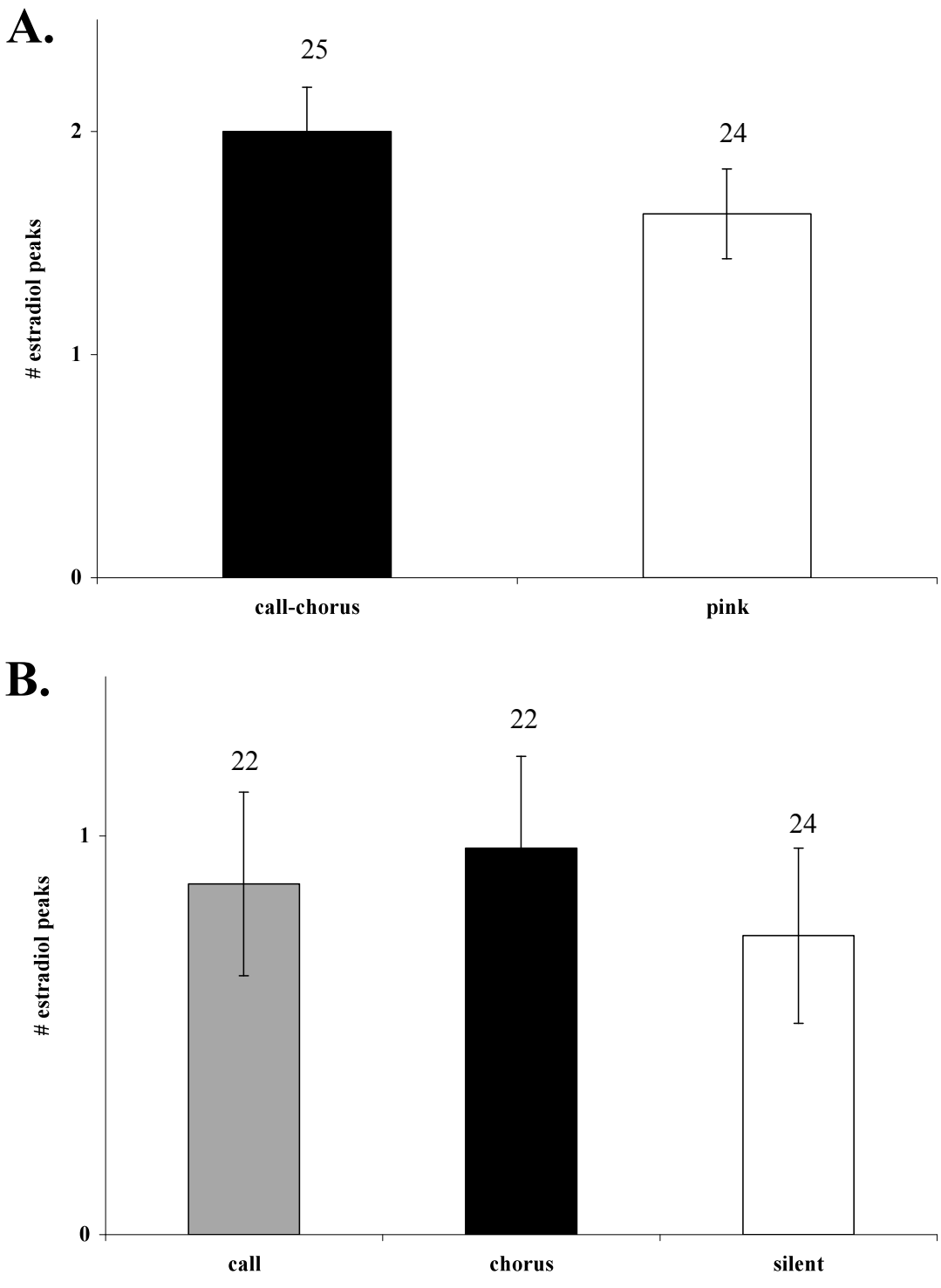


Figure 6: Mean ±S.E. levels of ovarian stage measured across two breeding seasons in females exposed to different acoustic treatments. (A) There was no significant difference in ovarian stages between females exposed to pink noise or call and chorus stimuli in 2005 (p=0.3010). (B) Ovarian stage was significantly less in females exposed to the silent treatment in 2006 compared to either of the conspecific stimuli (p=0.0221).

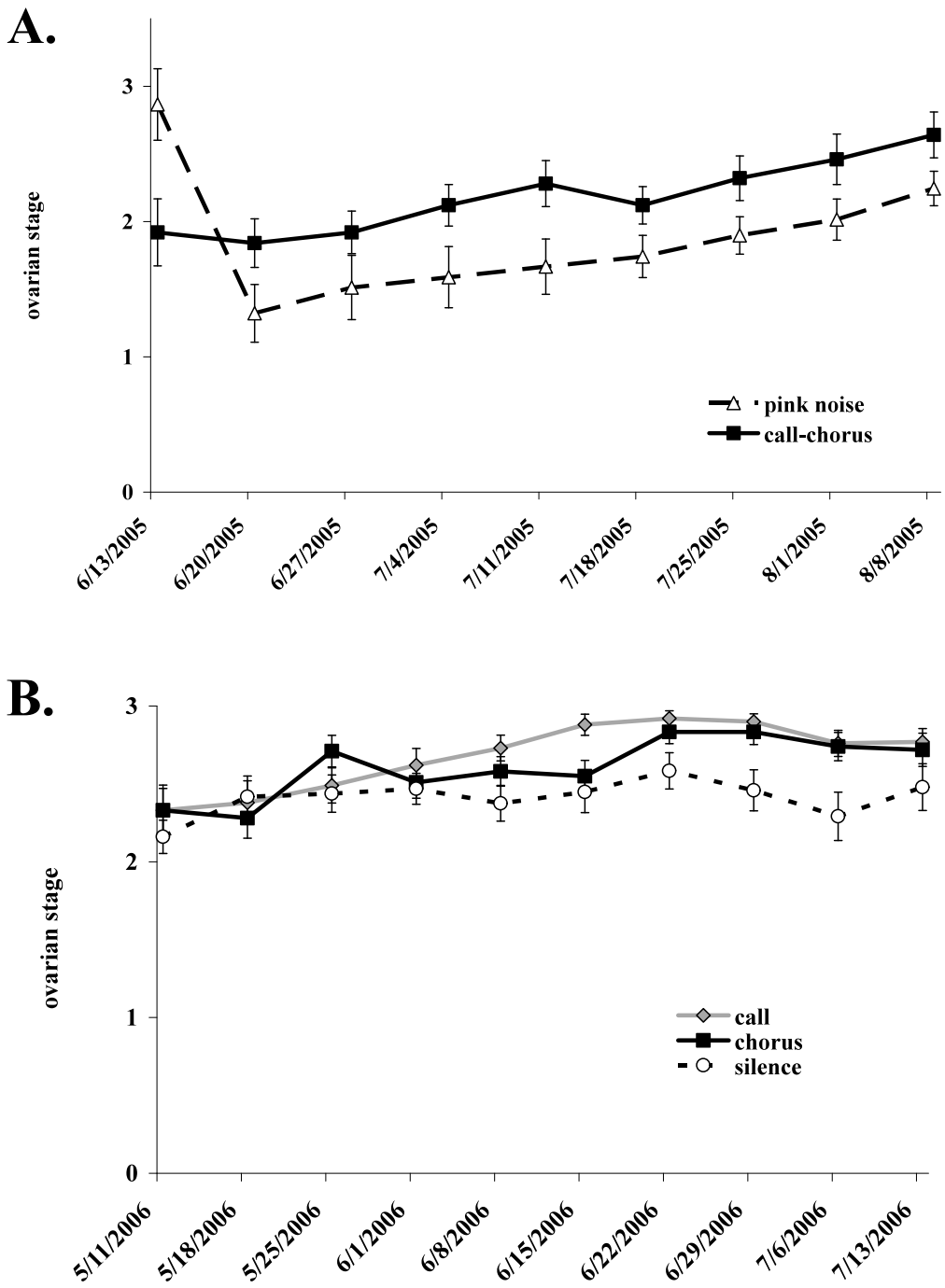


Figure 7: Mean ±S.E. of the number of weeks until stage III (when individual follicles are first detected in the ovaries) was observed in females exposed to different acoustic treatments. (A) Females exposed to pink noise took significantly longer to reach stage III than females exposed to call and chorus stimuli (*= p=0.0513). (B) There was no influence of acoustic treatment on ovarian development in 2006 (p=0.7827). Numbers above bars are sample sizes.

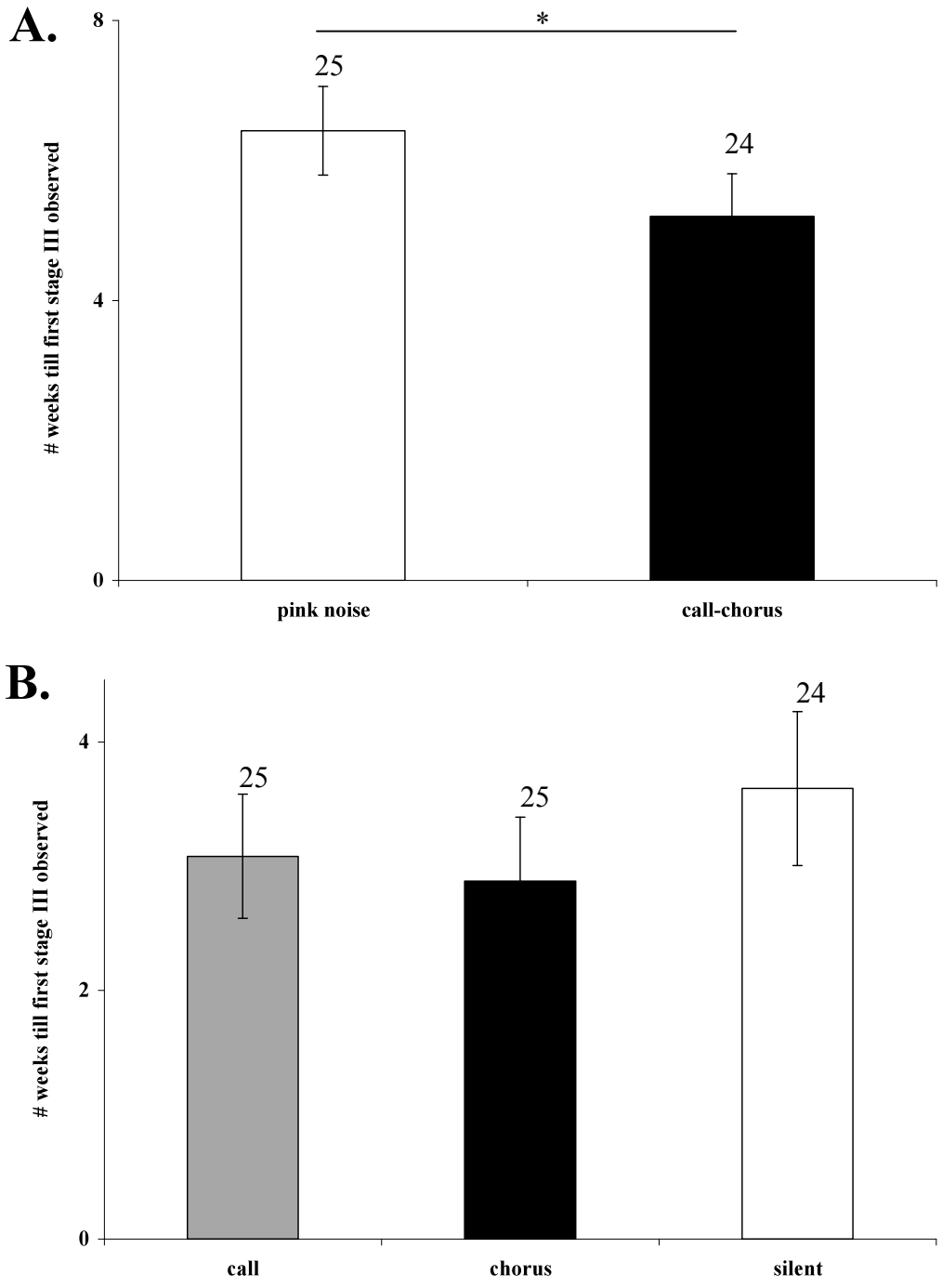


Figure 8: (A) Exposure to different acoustic treatments post brummation did not influence the likelihood of females exhibiting phonotaxis. (B) Mean ±S.E. response time of females exhibiting phonotaxis after post brummation stimuli exposure. Call and chorus noise exposed females took significantly longer to respond than females that had heard pink noise (p=0.0083). There was no significant difference in response time for females that heard call and chorus noise compared to the silent treatment (p=0.4697). Numbers above bars are sample sizes, *= significant at p<0.05.

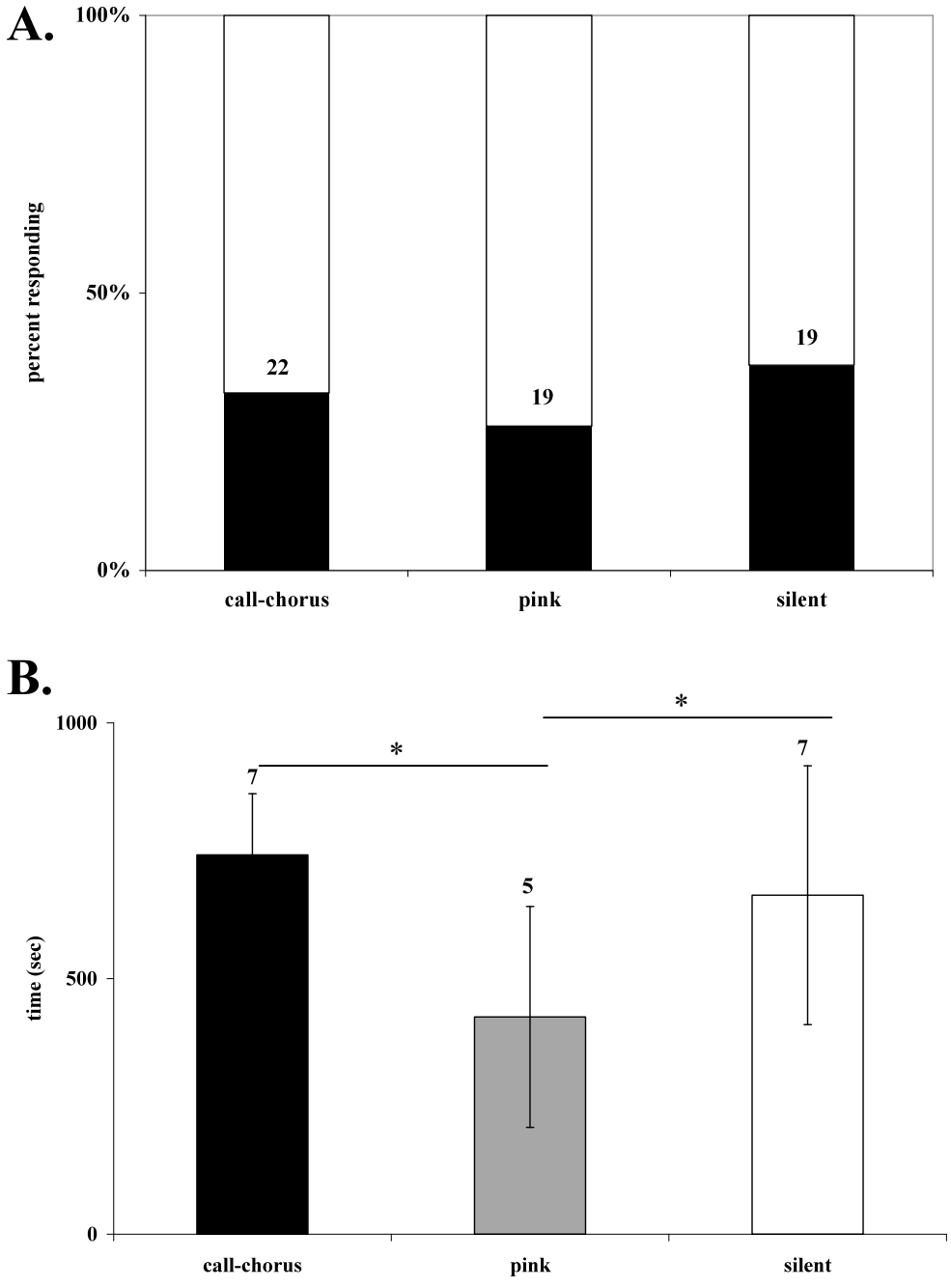
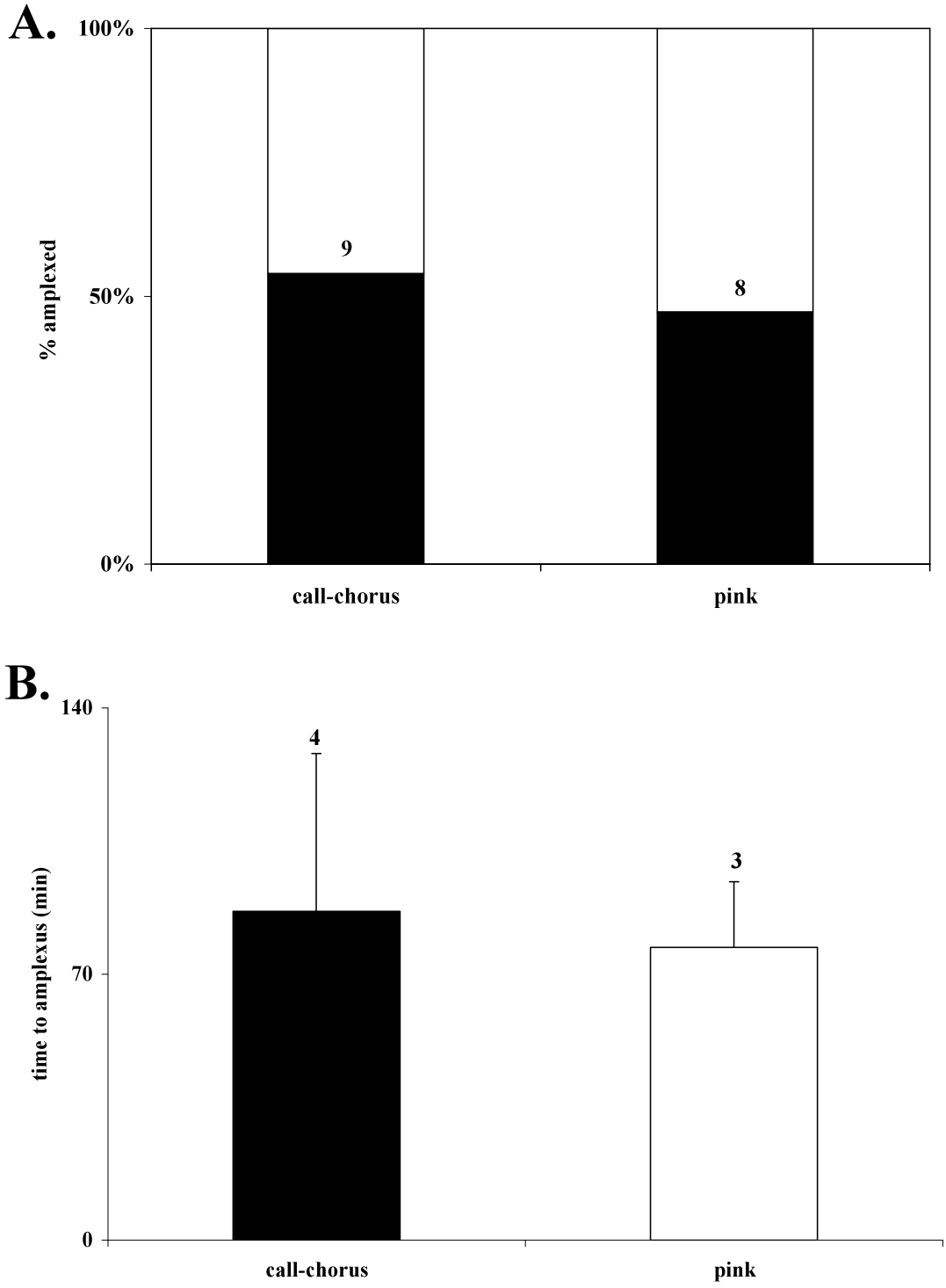


Figure 9: Exposure to different acoustic treatments post brummation did not influence (A) the likelihood of females exhibiting amplexus (p=0.7713) (B) or the time it took females to be found in amplexus (p=0.7291).



Chapter 5

Behavioral endocrinology of female gray treefrogs, *Hyla versicolor*, in response to acoustic stimulation: summary and general conclusions.

Noah M. Gordon

Patterns of reproductive steroids in gray treefrogs:

My work investigated the hormonal regulation of reproduction in female amphibians. I explored how hormones change in different seasonal and behavioral contexts. Here I summarize my findings to hypothesize a general schematic model for reproductive steroids in *Hyla versicolor* females and to call attention to how this pattern compares to patterns in other amphibians.

In female amphibians, reproductive steroids are produced primarily in the ovarian follicles (Redshaw 1972). The relative steroidogenic properties of the ovary are regulated by the proportions of follicles at different stages of development and not by the ovaries themselves (Fortune 1983; Kwon et al., 1991; Kwon et al., 1993), at least in the species where this has been measured (Iela et al., 1986; Degani et al., 1997). Since the number of advance-stage follicles is likely to be greatest at breeding events, it should be expected that plasma steroid levels will be greatest at this time as well. I found maximal levels of reproductive steroids at breeding in *H. versicolor* females (Gordon and Hellman, chapter 2) supporting this hypothesis in this species. Similar results have been reported

for *Pachymedusa dacnicolor* (Iela et al., 1986), a hylid treefrog with a breeding phenology similar to *H. versicolor*.

I found plasma levels of progesterone, testosterone and estradiol to be maximal on breeding nights, and all three steroids decreased dramatically within a single night of reproduction (Fig. 1). Progesterone levels declined most dramatically, reaching near-baseline levels prior to amplexus. Testosterone levels declined steadily from chorus arrival through oviposition, and estradiol levels did not begin to decline until after the initiation of amplexus or the beginning of oviposition (Gordon and Gerhardt, chapter 3). Estradiol levels in amplexed females did not reach near-baseline levels by the morning following oviposition, though both testosterone and progesterone levels did (Fig. 1). Because peak reproductive steroid levels were strongly indicative of breeding events, *H. versicolor* exhibits an associated reproductive pattern (sensu Crews and Moore 1986). The cycle of reproductive steroids in *H. versicolor* is similar to that of *Rana italica* (Guarino, et al., 1993) with the three steroids peaking just prior to reproduction, though in the latter species the progesterone peak is more prolonged than either the estradiol or androgen peaks.

Levels of reproductive steroids remain near baseline during periods between reproductive events in both captive and wild gray treefrogs. Recurring peaks of estradiol and progesterone in captive females (Gordon, chapter 4) suggest that steroid levels in females increase after a refractory period of 2-5 weeks, a period that encompasses the observed duration between clutches in *H. versicolor* (Gordon and Humfeld, unpubl.) and its sister species *H. chrysoscelis* (Roble 1985, Ritke and Lessman 1994).

Reproductive steroid levels in non-breeding female gray treefrogs vary with season. Background or baseline levels of estradiol and testosterone are greater during the breeding season (spring to early summer) compared to the non-breeding season, whereas baseline progesterone levels are greater during the non-breeding season (Fig. 2). Increased relative estradiol may reflect vitellogenic activity in the ovaries (Follet and Redshaw 1974). Seasonal patterns of androgens are correlated with ovarian development (Rastogi et al., 2005), though the exact nature of this relationship remains unclear. It is possible that the relative increase in progesterone in the non-breeding season is acting to inhibit initiation of a new ovarian cycle, as has been reported for *Salamandra salamandra* (Joly et al., 1994).

Influence of acoustic signals on female reproduction:

Male choruses of gray treefrogs occur on most nights during the breeding season and are audible several hundred meters away from breeding ponds where non-breeding females are found. Consequently, male acoustic signals would appear to be a likely option for females attempting to synchronize the timing of reproduction with male availability. Contrary to this hypothesis, my results do not suggest that females are using male acoustic signals to influence the timing of reproductive events (Gordon and Gerhardt, chapter 3; Gordon, chapter 4). Male signals did influence estradiol levels and the timing of oviposition on breeding nights (Fig. 3A), but females were already committed to oviposition at this juncture (Gordon and Gerhardt, chapter 3). Male signals may be acting to promote phonotaxis by increasing estradiol levels (Gordon and Gerhardt 2009). Further testing will be needed to determine if the increase in estradiol was

responsible for the observed difference in oviposition timing, or if the two events were merely correlated. The influence of male signals in delaying oviposition may not be biologically meaningful, since almost all females oviposit prior to sunrise, regardless of their acoustic environment (or even the presence of a male) (Gordon and Gerhardt, chapter 3).

The presence of an amplectant male had a strong influence on hormone levels in female treefrogs. Both estradiol and testosterone, but not progesterone, were elevated in the presence of an amplectant male (Fig. 3; Gordon and Gerhardt, chapter 3). This influence of amplexus was independent of acoustic treatment. It is unclear whether amplexus itself or a cue correlated with amplexus (e.g. visual, chemical or tactile cues) is responsible for the observed differences in estradiol and testosterone.

Influence of reproductive steroids on female phonotaxis:

Hormonal levels fluctuate during the breeding season in many anurans, but the identity of the hormones that modulate breeding behavior and their effects remain unclear. I found that female gray treefrogs treated with progesterone and prostaglandin exhibited phonotaxis to synthetic male advertisement signals significantly more often than did animals treated with ringers vehicle or uninjected controls, confirming the findings of Schmidt (1985). Responsive females had greater levels of plasma progesterone and estradiol compared to both control groups, suggesting that these steroids may be promoting phonotaxis (Gordon and Gerhardt, 2009). Furthermore, I found that the selectivity of hormonally-induced phonotaxis in *H. versicolor* was similar to that observed in freshly captured breeding animals. Females made the same choices

between acoustic signals after hormone treatments in tests of frequency, call rate and pulse rate, compared to their responses without treatment immediately after collection from the breeding chorus. The preference for a longer call was, however, significantly weaker after hormone induction of phonotaxis. Hormonally primed females were also less likely to respond in any test and took longer to respond than did freshly collected females (Gordon and Gerhardt 2009). Consequently, my study shows how progesterone-prostaglandin induced phonotaxis in female treefrogs influences both the quality and quantity of phonotaxis, relative to that exhibited by naturally breeding females.

General conclusions:

My work is the first to show the timescales over which acoustic signals may influence female physiology in frogs. This is the first study to demonstrate a relationship between steroids and oviposition timing in frogs. That acoustic signals influenced oviposition in an unexpected direction is a highlight of my work. Furthermore, I have increased our understanding of the hormonal regulation of phonotaxis behavior and amplexus in anurans. Overall my research contributes to a greater understanding of the mechanisms regulating reproduction in amphibians.

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FIGURES

Fig. 1: Schematic of representation of reproductive steroid cycle on breeding nights for female *Hyla versicolor*. Pre- and post-breeding levels are for females away from breeding choruses, remaining values are for females at, or approaching, breeding choruses. A single reproductive event from approaching male choruses to postoviposition occurs within a single night.

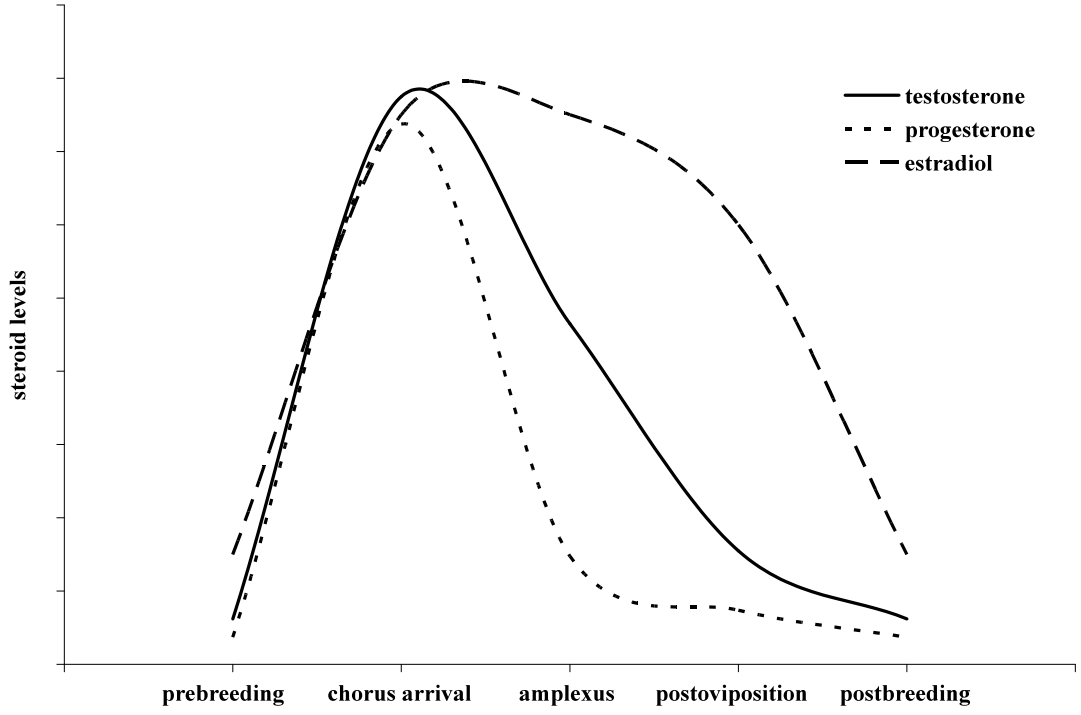


Fig. 2: Schematic representation of annual reproductive steroid cycle for female *Hyla versicolor* with two hypothesizes breeding events (hormonal peaks). The breeding season lasts from spring to early summer.

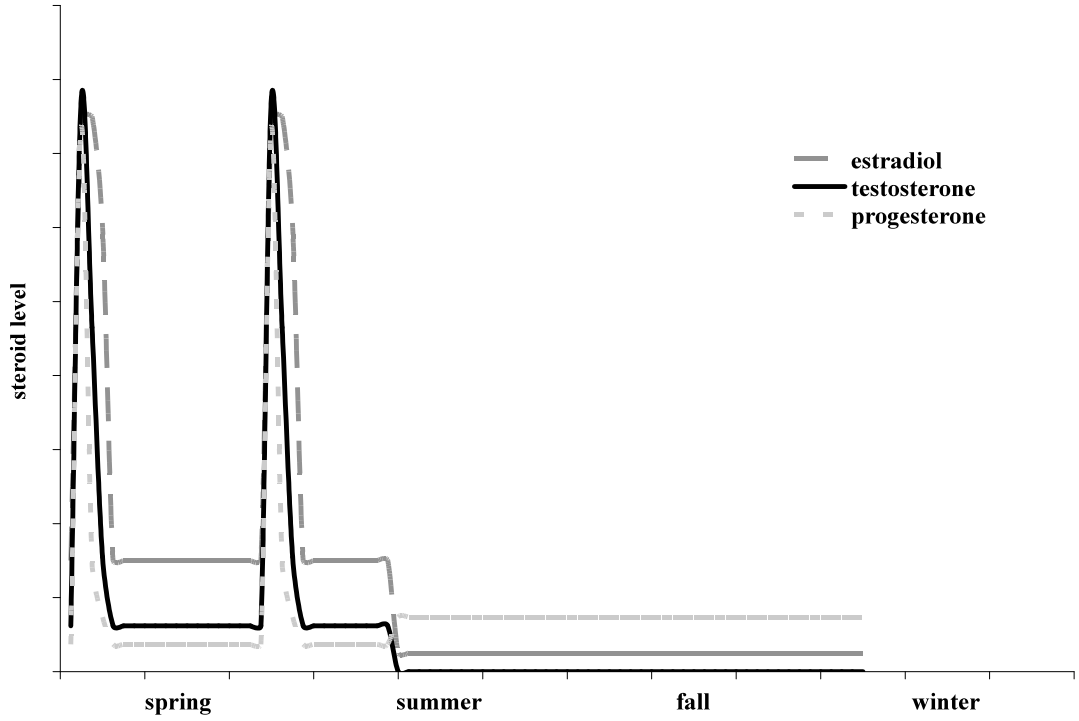
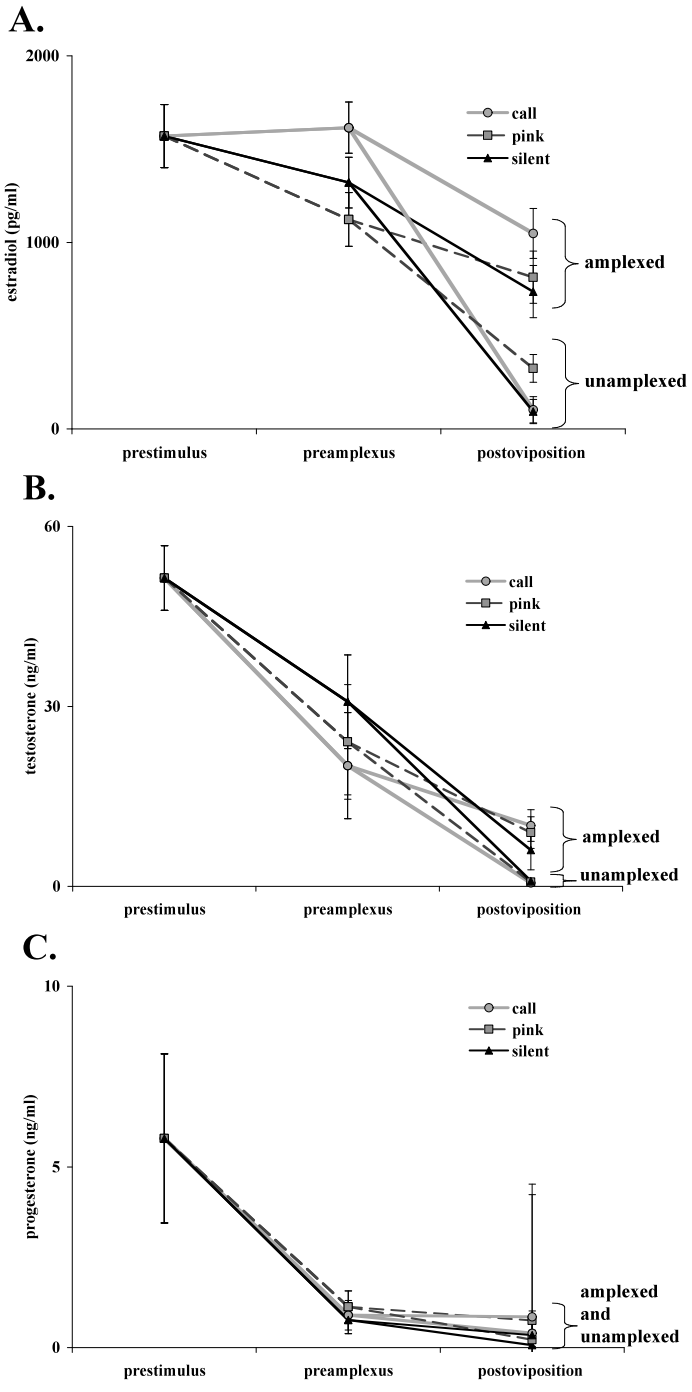


Fig. 3: Influence of acoustic treatment (conspecific calls, pink noise, or silence) and amplexus on steroid levels in *Hyla versicolor* females: (A) estradiol, (B) testosterone and (C) progesterone.



VITA

Noah Gordon was born October 22, 1968 in Philadelphia, Pennsylvania. He went to Montessori kindergarten where he was hit in the head with a rock giving him a scar that is visible to this day. It was around this time that he caught his first salamanders (*Plethodon cinereus),* toads (*Bufo americanus*) and snakes (*Storeria dekayii*). He attended a mixture of public and private schools, moved to Norwich, Connecticut with his family, and eventually graduated from Norwich Free Academy in 1986. Noah then went to the University of Connecticut where he was an excellent procrastinator, eventually graduating in 1998 with a B.G.S, which is the degree they give you when you've been around so long that they have to give you something. When he was not in school, Noah spent several years operating a camping/outdoors store and working with autistic adults. Toward the end of his long and distinguished undergraduate career, he realized that a career playing with herps was a possibility. After hanging around with EEB grad students for a few years, Dr. Kent Wells gave him a chance at UConn, where Noah earned his M.S. in 2001. While a graduate student at UConn, Noah passed out in front of his class while teaching introductory biology, giving him another scar to match the one from kindergarten. He moved to Ohio, where two years as a lab tech at Miami University introduced him to his eventual wife, and they both moved to Missouri where Noah earned his PhD in the lab of Dr. Carl Gerhardt in 2008. He will begin a postdoc at the University of Notre Dame in the lab of Dr. Sunny Boyd in 2009, after which he hopes to find a tenure-track position. RLF.