

## Experimentally induced sexual behavior in male gray treefrogs activates the HPG but not the HPI axis

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### ABSTRACT

While many studies have established how hormones modulate behavior, experiments that manipulate a behavior and quantify its hormonal consequences are rarer. Frog sexual behavior presents an opportunity to control a discrete behavior and observe its secretory consequences. Male frogs clasp females (amplexus) prior to gamete release. In the wild, amplexed males have endogenously higher gonadal and interrenal steroids. Here, we used Cope's gray treefrogs (*Hyla chrysoscelis*) to experimentally test the hypothesis that the behavioral act of amplexus causes these increased circulating hormones. We quantified levels of testosterone, estradiol, and corticosterone after 90 min in three treatment groups: unpaired males that were given access to a female and allowed to enter amplexus ("amplexus induced"), males in naturally amplexed pairs that were separated from their mate ("amplexus terminated"), and unpaired males that remained unpaired ("control"). Testosterone and estradiol were elevated in the amplexus induced group relative to males in the amplexus terminated and control groups. We did not observe any differences in corticosterone levels across treatment groups. These results suggest the behavioral act of amplexus activates the hypothalamic-pituitary-gonadal axis, but not the hypothalamic-pituitary-interrenal axis. This study provides experimental evidence of a discrete behavior rapidly initiating hormonal changes.

### 1. Introduction

That hormones both modulate and respond to behavior is well-established (Alward et al., 2018; Wingfield et al., 2019). While investigating the latter directionality is challenging due to the need to reliably manipulate behavior, there have been many studies identifying secretory changes related to reproduction and social competition. Cheng (1986, 1992), for example, discovered that the combined sensory inputs of female ring doves hearing their own coos and the head-down posture of vocal production activate the hypothalamic-pituitary-gonadal (HPG) axis, which then triggers egg laying. Studies on the Challenge Hypothesis (Wingfield et al., 1990) have shown aggressive encounters can increase androgens (Wingfield et al., 2019), which can in turn prime animals to win future fights (Fuxjager et al., 2011). Along with the HPG axis, activity in the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis is also upregulated during the breeding season in amphibians, reptiles, and birds to support energy mobilization during mating and reproduction (Moore and Jessop, 2003; Romero, 2002).

Frog reproduction presents an excellent opportunity to examine

hormonal responses to behavior. Before mating, frogs typically first attract a mate by producing advertisement calls. The Energetics-Hormone Vocalization hypothesis proposes androgens maintain the high vocal efforts male frogs use to attract females, but due to energetic demands of calling, glucocorticoids should increase until they inhibit androgens, which should reduce calling (Emerson, 2001). Relationships between androgens, glucocorticoids, and calling effort in male frogs, however, do not always support this hypothesis (Leary et al., 2015). Unlike calling, which is highly variable, amplexus permits investigation of a discrete behavior and its secretory consequences. In many frog species, mating occurs only after the male performs the simple, but energetically demanding task of clasping the female in amplexus (Fig. 1), which often lasts for several hours before fertilization (McLister, 2003). In many amphibians, steroid hormone levels are higher in amplexed males compared to unpaired males (Harvey et al., 1997; Orchinik et al., 1988; Reedy et al., 2014). What causes elevated hormones in amplexus, however, is unknown.

Similar to other amphibians, males of Cope's gray treefrog (*Hyla chrysoscelis*) collected in amplexus have higher levels of estradiol (E<sub>2</sub>),

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**Fig. 1.** Photograph of a male (right) and female (left) of Cope's gray treefrog (*Hyla chrysoscelis*) in amplexus in the animals' natural environment. (Photo courtesy of Norman Lee.)

tosterone (T), and corticosterone (CORT) compared to unpaired calling males (Baugh, 2024). In this study, we investigated the causal relationship between amplexus and circulating steroid hormones in male *H. chrysoscelis*. To test the hypothesis that the behavioral act of amplexus elevates male hormone levels, we compared  $E_2$ , T, and CORT levels after 90 min across three groups of males that were either induced to enter amplexus, removed from amplexus, or prevented from entering amplexus.

## 2. Methods

### 2.1. Animals

We collected *H. chrysoscelis* from the Carver Park Reserve (Carver County, MN, USA, 44°52'49.29" N, 93°43'03.10" W) between May 23 and June 8, 2024. We searched ponds beginning at 2200 h using headlamps or lanterns until we found an amplexed pair. Within 5 min of collecting the pair, we collected two unpaired calling males. Frogs (168 total:  $n = 42$  amplexed males, 42 amplexed females, 84 unpaired males) were placed in plastic containers (14.5 cm  $\times$  10.2 cm  $\times$  7.5 cm) and transported 60 m away from the chorus to a processing station. All animals were handled, and all procedures were conducted, following international guidelines and protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

### 2.2. Amplexus experiment

Each experimental trial used the amplexed pair and two unpaired males. Most trials (93 %) began before 2330 h. Three clear plastic containers (12.5 cm  $\times$  12.5 cm  $\times$  6.3 cm) with a thin layer of pond water housed individuals during trials. We separated the amplexed male from the female and placed it into a container alone ("amplexus terminated"). We placed the female with a randomly chosen unpaired male ("amplexus induced"). Females removed from amplexus readily re-enter amplexus (Love and Bee, 2010). Lastly, we placed the second unpaired male alone in the third container ("control"). If amplexus did not occur in the new pair within 25 min ( $n = 6/42$  trials), we used another unpaired male collected within 30 min of the first. If this pair never entered amplexus, we aborted the trial. Once the new pair entered amplexus, we left frogs undisturbed in their containers for 90 min to allow time for steroid hormone levels to respond to treatment without enough time for fertilization to occur (Orchinik et al., 1988). We wore headlamps with dim red light to minimize light exposure.

### 2.3. Blood sampling

After the 90-min experiment, we collected whole blood (20–80  $\mu$ L) within 5 min (mean  $\pm$  SD = 2.4  $\pm$  1.4 min, range = 1–7 min measured to nearest minute) of handling each frog via cardiac puncture (Baugh, 2024), using a 30-gauge insulin syringe (BD Micro-fine U-100, 0.3 mL, Franklin Lakes, NJ). We kept whole blood samples on ice for 1–4 h before transportation to the lab, where they were centrifuged at 8  $^{\circ}$ C at 7500 RPM for 8 min (Eppendorf 5418, Hamburg, Germany) to separate plasma. We stored plasma at  $-20^{\circ}$ C for 4–6 weeks before shipping it on dry ice to Swarthmore College to be kept at  $-80^{\circ}$ C. Single males were released and the newly amplexed pair was returned to the University of Minnesota for other behavioral experiments.

### 2.4. Steroid extraction and dilution

All protocols have been validated (Baugh, 2024; Gall et al., 2019). We used a diethyl ether double extraction method (see Baugh et al., 2012 and Baugh, 2024 for further details). All available plasma (8–74  $\mu$ L) was used. Following extraction, we froze samples at  $-80^{\circ}$ C for 1–2 days or we resuspended immediately and allowed samples to reconstitute for 12 h at 4  $^{\circ}$ C. We resuspended samples in assay buffer from enzyme immunoassay (EIA) kits. In males, optimal reconstitutions are at 1:10 for  $E_2$ ; 1:80 for T; and 1:10 for CORT (Baugh, 2024; Gall et al., 2019). Information on steroid extraction and quantitation of female plasma samples is outlined in Appendix A.

### 2.5. Enzyme immunoassays

Following Baugh (2024), we estimated steroid concentrations using EIA kits (DetectX<sup>®</sup> kits, Arbor Assays, Ann Arbor, MI) for 17 $\beta$ -estradiol (Cat. No. KB30, Donkey anti-Sheep IgG), testosterone (Cat. No. K032, Goat anti-Rabbit IgG), and corticosterone (Cat. No. K014, Donkey anti-Sheep IgG). We lost one CORT plate due to a reagent sequence error. We measured  $E_2$  in 115 males (25 amplexus induced, 36 amplexus terminated, 39 control), T in 123 males (43 amplexus induced, 40 amplexus terminated, 40 control), and CORT in 83 males (25 amplexus induced, 30 amplexus terminated, 28 control). We analyzed samples following the manufacturer's instructions. We plated most samples (87 %) in duplicate and low volume samples as singlets ( $n = 19$  for  $E_2$ ,  $n = 12$  for T,  $n = 10$  for CORT). We used an internal standard (pooled *H. chrysoscelis* plasma) for calculation of intra- and inter-assay coefficients of variation (CV). One T sample with a high CV (100 %), likely resulting from human error, was not analyzed. If one duplicate sample fell below the detection limit, we used the measurable singlet ( $n = 2$  for  $E_2$ ,  $n = 1$  for T,  $n = 7$  for CORT). If both samples fell below detection, we estimated concentration as halfway between zero and the detection limit ( $n = 0$  for  $E_2$ ,  $n = 0$  for T,  $n = 3$  for CORT). Please refer to the Appendix for detection limits, cross-reactivity, and CVs.

### 2.6. Statistics

We performed analyses in R v 4.4.1 (R Core Team, 2025) using  $\alpha = 0.05$ . We analyzed treatment effects on steroid hormone concentrations using linear mixed models with the *lme4* package (Bates et al., 2014) with trial included as a random effect to control for day and time of night of each trial. We confirmed no confounding covariates explained additional variation in steroid hormone levels (see Appendix A). We square-root transformed T and  $E_2$  levels, and log<sub>10</sub>-transformed CORT levels to improve normality of residuals. Outliers ( $n = 0$  for  $E_2$ ,  $n = 3$  for T,  $n = 2$  for CORT) were defined as any point above  $Q3 + 1.5 \times IQR$  or below  $Q1 - 1.5 \times IQR$  (Tukey, 1977) and removed. Outlier inclusion or removal did not affect interpretations (Fig. A.1). We found no model violations after assessment with the *DHARMA* package (Hartig, 2022). We ran post hoc tests using estimated marginal means and report effect sizes as Cohen's *d* using the *emmeans* package (Lenth, 2023). We also assessed

correlations between hormones levels in both males and females and between males and females. Details on the analysis and results from these correlations can be found in Appendix A.

### 3. Results

Gonadal steroid levels varied significantly as a function of experimental treatment ( $E_2$ :  $\chi^2(2,114) = 20.335$ ,  $p < 0.001$ , Fig. 2A; T:  $\chi^2(2,118) = 15.165$ ,  $p < 0.001$ , Fig. 2B). Both  $E_2$  and T were highest in the amplexus induced group, intermediate in the amplexus terminated group, and lowest in the control group (Fig. 2A, B). Compared to control males, levels of  $E_2$  and T in the amplexus induced group were significantly different, averaging 34 % ( $E_2$ :  $p < 0.001$ , Cohen's  $d = 1.023$ ; Fig. 2A) and 25.2 % (T:  $p < 0.001$ , Cohen's  $d = 0.869$ ; Fig. 2B) higher, respectively.  $E_2$  levels were also significantly higher, by 25.0 % on average, in the amplexus induced group compared to the amplexus terminated group ( $p = 0.038$ , Cohen's  $d = 0.583$ ; Fig. 2A). T levels in the amplexus induced group averaged 14.7 % higher than in the amplexus terminated group, but this difference was not significant ( $p = 0.057$ , Cohen's  $d = 0.529$ ; Fig. 2B). Compared with control males,  $E_2$  and T levels in the amplexus terminated group were somewhat higher (7.2 % and 9.1 %, respectively), but these differences were not significant ( $E_2$ :  $p = 0.159$ , Cohen's  $d = 0.440$ , Fig. 2A; T:  $p = 0.298$ , Cohen's  $d = 0.339$ , Fig. 2B).

In contrast to the two gonadal steroids, CORT levels did not differ significantly based on experimental treatment ( $\chi^2(2,80) = 0.638$ ,  $p = 0.727$ , Fig. 2C). Compared to control males, CORT levels were 1.4 % higher in the amplexus induced group ( $p = 0.949$ , Cohen's  $d = 0.086$ ) and 3.2 % higher in the amplexus terminated group ( $p = 0.710$ , Cohen's  $d = 0.214$ ). CORT levels were 1.8 % higher in the amplexus terminated group compared to the amplexus induced group ( $p = 0.892$ , Cohen's  $d = 0.128$ ).

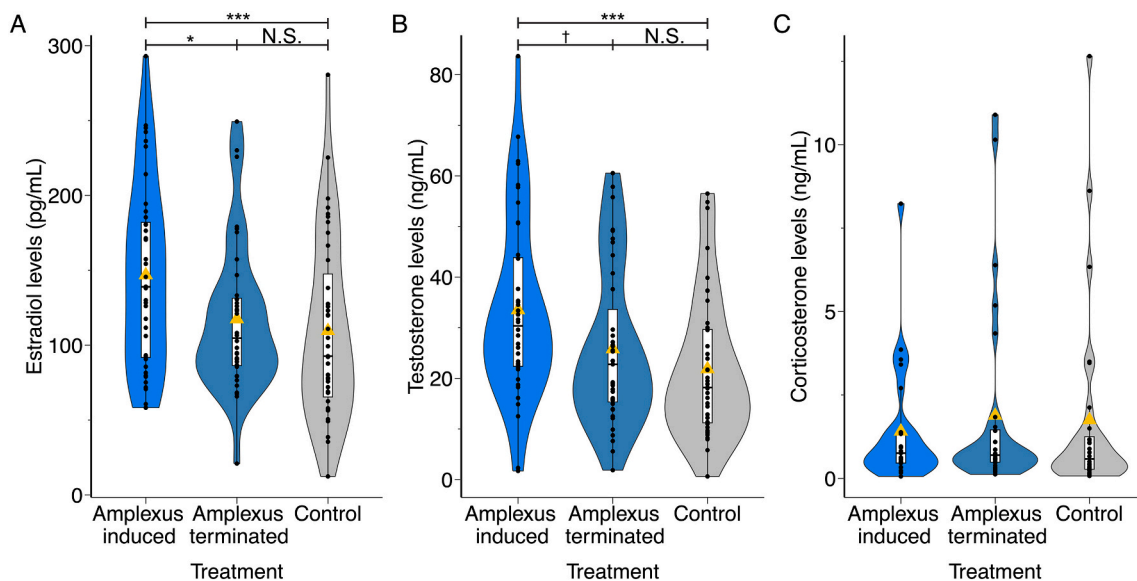
### 4. Discussion

We found evidence in male frogs supporting the hypothesis that amplexus with a female increases secretory activity for  $E_2$  and T, but not CORT. These results are consistent with other studies demonstrating

elevated gonadal steroid levels in amplexed male amphibians, including *H. chrysoscelis* (Baugh, 2024; Harvey et al., 1997; Orchinik et al., 1988). We suggest amplexus behavior is likely causally related to gonadal steroid hormone production, with one important caveat. This conclusion must remain provisional because our study design does not allow us to reject the alternative hypothesis that mere sensory detection of a female is the sole or a contributing inducer.

Rapid endocrine responsiveness to mating opportunity is likely adaptive. Enhanced activity in the HPG axis triggered by the act of amplexus likely facilitates mating as androgens might increase skeletal muscle tone in trunk and arms used for clasping. For example, in bullfrogs, T supports enhanced clasping force in the forelimb muscles (Kampe and Peters, 2013). Amplexus-induced HPG activity may also promote sperm release (Silla et al., 2019). Studies have linked changes in circulating  $E_2$  to female reproductive behaviors (Cheng, 1986, 1992) and central action of  $E_2$  can promote non-breeding aggression and enhance sensory responsiveness to social stimuli in males (Remage-Healey et al., 2010; Silva et al., 2020). Elevated  $E_2$  in mating males is a novel and possibly paradoxical finding. Mechanistically, the increased  $E_2$  might simply be driven by testicular aromatization of the high T in amplexed males (Miano et al., 1999; Urbatzka et al., 2007). Functionally, however, experimentally elevated  $E_2$  in *Xenopus laevis* has largely been shown to inhibit male calling (e.g. shifting away from courtship calling; Hoffmann and Kloas, 2012b), which occurs through estrogen receptor signaling (Hoffmann and Kloas, 2012a). On the one hand, this is consistent with amplexed males' calling cessation; on the other hand, the simultaneously elevated T should have precisely the opposite effect. One hypothesis to be tested in a future pharmacological manipulation is that elevated plasma  $E_2$  is sufficient to counteract the otherwise stimulatory effect of T on calling in vocal-motor areas of the brain, while high systemic T levels act to drive spermatogenesis and sustained motor activity for amplexus in the periphery.

Unlike the HPG axis, we did not observe elevated CORT following 90 min of induced amplexus, in contrast to what has been observed in naturally amplexed male anurans (Baugh, 2024; Harvey et al., 1997; Orchinik et al., 1988; Reedy et al., 2014). In marine toads, CORT appears to peak after approximately 3 h of continuous amplexus (Orchinik et al., 1988). If a similar timeline were present in *H. chrysoscelis* males, our



**Fig. 2.** Gonadal steroid levels, but not CORT, increased in males in response to amplexus. Violin plots depict (a)  $E_2$ , (b) T, and (c) CORT levels after 90 min in males in the amplexus induced group (royal blue), the amplexus terminated group (steel blue), and the control group (gray). Black horizontal lines inside boxplots represent the median and whiskers extend to 1.5 x IQR. Yellow triangles represent the mean. Steroid levels were analyzed statistically after square-root transformation ( $E_2$ , T) or  $\log_{10}$  transformation (CORT), but are shown without transformation. Hormone levels depicted with transformation can be found in the Appendix (Fig. A.2). \*\*\*,  $p < 0.001$ . \*,  $p < 0.05$ . †,  $p = 0.057$ . N.S., not significant.

sampling timeline would have missed this slower response. Alternatively, if the HPA axis is rapidly activated by the initiation of amplexus—as in an acute stress response—rather than the maintenance of amplexus, it is possible that CORT levels had declined back to baseline following negative feedback at our 90 min sampling time point. For example, 90 min is sufficient to observe a return to baseline (and even sub-baseline) CORT levels following acute stressors in songbirds (Baugh et al., 2017).

We cannot rule out that sexual selection might also contribute to higher gonadal steroid levels in naturally amplexed males (Leary and Knapp, 2014). Female *H. chrysoscelis* prefer males with longer and louder calls produced at faster rates (Bee et al., 2012; Tanner et al., 2017). While androgens can stimulate call production, the influence of a male's hormone profile on graded vocal performance remains unclear (reviewed in Leary, 2009 and Leary and Baugh, 2020; Leary and Harris, 2013). It is, therefore, conceivable that males with naturally elevated hormones are more attractive. Indeed, the intermediate hormone levels observed in amplexus-terminated males in the present study might be the result of such a sexual selection bias. Alternatively, those intermediate hormone levels might be the result of declining hormone levels caused by termination of the earlier act (or experience) of amplexus.

Our results suggest a discrete mating behavior can activate secretion of the two primary endocrine products of the HPG axis in male *H. chrysoscelis*. Future experimental studies will be necessary to distinguish between the potential causal roles of performing motor behaviors (e.g. forearm, trunk muscle activity), the proprioceptive feedback that motor activity generates, and sensory stimulation from the female (e.g., tactile, visual, olfactory). Our results confirm that the elevated levels of gonadal steroid hormones previously observed in amplexing amphibians is likely caused by the act of mating itself. These findings provide another compelling example of how behavior can trigger changes in endocrine activity and highlight a potential novel role of estradiol in reproduction in male amphibians.

#### CRediT authorship contribution statement

**Megan K. Freiler:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liam N. Halstead:** Methodology, Investigation, Data curation. **Mark A. Bee:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Alexander T. Baugh:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2025.105864>.

#### Data availability

The associated data are available at the Data Repository of University of Minnesota (DRUM): <https://doi.org/10.13020/zy6y-tq15>.

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