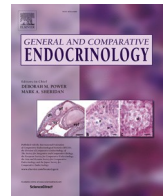


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Male Cope's gray treefrogs (*Hyla chrysoscelis*) in amplexus have elevated and correlated steroid hormones compared to solitary males

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ABSTRACT

Gonadal steroid hormones are typically elevated during the breeding season—a finding known as an associated reproductive pattern. Though less studied, there is also evidence, in both sexes, for elevated adrenal/interrenal steroids, including acute elevations on the day of mating. I investigated gonadal and interrenal steroids in wild male Cope's gray treefrogs at breeding aggregations. I collected blood from males found in amplexus with female mates (amplexed males) and males sampled at the same time and location that were actively advertising vocally and without a mate (solo males). Concentrations of plasma corticosterone, testosterone, and 17β -estradiol (CORT, T and E_2 , respectively) were validated and measured. These two categories of males differed in four ways: (1) amplexed males exhibited significantly elevated concentrations of all three steroids compared to solo males (CORT: +347 %; T: +60 %; and E_2 : +43 %); (2) these hormone profiles alone accurately predicted male mating category with ca. 83 % accuracy using a discriminant function analysis; (3) amplexed males exhibited significant between-hormone correlations (T and E_2 were positively correlated and CORT and E_2 were negatively correlated) whereas no correlations were found in solo males; (4) amplexed males showed a negative correlation with CORT concentration and the time of night, whereas no such pattern was present in solo males. These findings suggest an acute and strong coactivation of the interrenal and gonadal axes that could drive phenotypic integration during this fitness-determining moment. I discuss these findings and suggest experiments to determine causation, including the role of motor behavior driving endocrine states and the role of female selection on endocrine profiles.

1. Introduction

Much research has investigated endocrine mechanisms that mediate the transition from non-breeding to breeding condition across seasons, and the behaviors that accompany this transition, with an emphasis on the activation of the hypothalamic-pituitary-gonadal (HPG) axis (reviewed in [Adkins-Regan, 2005](#)). There can also be a more acute transition, particularly for males in many species in which mating opportunities are rare and unpredictable. For example, in anurans (frogs and toads), males compete for females under highly male-skewed operational sex ratios with a low probability of mating on a given night ([Ryan, 1985](#); reviewed in [Gerhardt and Huber, 2002](#)). If selected by a female, the male clasps the female in amplexus for a period of time typically lasting several hours (e.g. 10 h in *Hyla versicolor*; [McLister, 2003](#)) and ending with female oviposition and male fertilization. Amplexus is energetically demanding ([Ryan et al., 1983](#); [Ryan, 1985](#); [McLister, 2003](#)), especially for the forearm muscles in males ([Kampe and Peters, 2013](#)), and this period of time provides males the opportunity to

spermiate in preparation for external fertilization (reviewed in [Méndez-Tepepa et al., 2023](#)). Hence, this amplexus window likely involves rapid endocrine changes required to support spermiation (via enhanced activity in the HPG axis), as well as a shift from the aerobic demands specific to vocalizing to those required for clasping. In eastern gray treefrogs (*H. versicolor*), vocalizing involves a higher absolute metabolic increase above resting metabolism owing to the larger muscle mass experiencing aerobic activity; clasping, on the other hand, demands a relatively higher aerobic respiration per gram of muscle tissue ([McLister, 2003](#)). Like other metabolic transitions, the shift from vocalizing to clasping might be accompanied by changes in secretory activity of the hypothalamic-pituitary-adrenal/internal (HPA/I) axis. The involvement of the HPA/I axis in mediating reproduction has been understudied compared to the HPG axis (reviewed in: [Sapolsky et al., 2000](#); [Carr, 2011](#); [Toufexis et al., 2014](#)), though its involvement appears to be widespread in vertebrates and key to life history transitions that demand rapid reprioritization of limited glucose stores ([Wingfield and Sapolsky, 2003](#); [Cotton et al., 2006](#); [Breuner et al., 2008](#); [Tokarz and Summers,](#)

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2011), and hence plays an important role in resolving tradeoffs such as between reproduction and immunocompetence (Titon et al., 2016, 2021).

One major avenue of investigation has centered on the environmental and social cues that induce endocrine activity that prepares animals for mating (reviewed in Moore et al., 2005). This line of research has shown, among other findings, that environmental cues such as day length, precipitation and phenological attributes are important for activating the HPG axis, which is followed by maturation of ova and sperm (Nelson, 2005; Vu and Trudeau, 2016; Silla et al., 2019). Other studies have focused on social cues, including the visual, tactile and acoustic presence of conspecifics as the cues (reviewed in Adkins-Regan, 2005). For example, it has been shown that exogenous testosterone can increase courtship behavior (Alward et al., 2017; Fuxjager et al., 2013; Ramage-Healey and Bass, 2006) and that social cues such as receptive mates and competitors can induce increases in plasma testosterone (Gleason et al., 2009; Goymann et al., 2019). Likewise, estradiol can have rapid effects of male behavior such as aggression (Trainor et al., 2008), and estrogen receptors are located throughout areas of the brain that mediate social and sexual behavior, including in male anurans (Chakraborty and Burmeister, 2010). Because many previous studies have examined a single steroid (e.g. testosterone) or axis (e.g. HPG), it is unknown whether concomitant changes in other hormones or axes, and their interactions, are responsible for observed changes in acute sexual readiness. Moreover, the focus on sensory cues that potentiate secretory changes promoting sexual readiness has largely ignored the possibility that the motor behaviors triggered in response to those sensory cues are critical to the observed endocrine changes. Reciprocal interactions between hormones and behavior are challenging to investigate and require a system well suited to such questions.

Male frogs and toads present an excellent opportunity to begin investigating how motor behaviors might trigger endocrine activity which then subsequently feeds back on sexual readiness (reviewed in Moore et al., 2005). One of the few experimental studies on this topic was conducted by Orchinik et al. (1988), which showed that male marine toads (*Bufo marinus*) in amplexus exhibited elevated androgens and glucocorticoids compared to solo males. This effect was demonstrated both in field collected males as well as a captive experiment whereby solo males were randomly assigned to be in amplexus with a female or not (solo). Males in amplexus had significantly elevated corticosterone and testosterone relative to solo males. One interpretation of these results is that the experience of engaging in amplexus behavior in male *B. marinus* causes elevated corticosterone and testosterone secretion over a relatively fast time frame (<2 h). This finding was interpreted in the context of the explosive (compared to prolonged; Wells 2007) breeding behavior in *B. marinus*, which coordinate breeding activity on a small number of nights. The idea was that because the toads must successfully reproduce on a single night, they will (a) rapidly activate their gonadal endocrine axis via proximate cues (presence of a female or amplexus) indicating demand; and (b) that reproduction in the toads would be uninhibited by elevated glucocorticoids because of the need to proceed with sex during these acute, discrete opportunities.

A few questions emerge from this observation. First, is this a general phenomenon in other species, including those that are not explosive breeders? This is important because rapid activation of the gonadal axis via acute sensory cues or behavioral acts (or both) might be a more general mechanism underlying rapid transitions to sexual readiness, including in prolonged temperate breeders; and buffering of sexual inhibition from GCs appears to be more widely applicable and in fact key to the energetic transition required during successful mating behavior (reviewed in Leary and Baugh, 2020). Second, are other gonadal products such as estrogens implicated? Third, how are gonadal and adrenal/interrenal steroids correlated within individuals and does coactivation of gonadal and interrenal secretion via amplexus drive correlations? Lastly, how do other variables, including the timing of amplexus, influence endocrine changes? In the present study I investigate these four

areas using Cope's gray treefrogs (*Hyla chrysoscelis*), a prolonged breeding species, at natural breeding aggregations and sampled for corticosterone, testosterone, and 17 β -estradiol (hereafter CORT, T, and E₂, respectively).

2. Materials and methods

2.1. Animals and blood sampling

I collected males of the western genetic lineage of Cope's gray treefrog (Booker et al., 2022) from wetlands located in the Carver Park Reserve (Carver County, MN, USA, 44°53'49.08" N, 93°43'03.11" W, 302 m elevation) and the Tamarack Nature Center (Ramsey County, MN, USA, 45°06'08.50" N, 93°02'28.89" W, 279 m elevation) on four nights between the 27th of May through the 6th of June in 2022. The Tamarack site hosts syntopic populations of *H. chrysoscelis* and the cryptic sister species, *H. versicolor*. Therefore, following blood collection, I confirmed that all males in this study were *H. chrysoscelis* by permitting them to vocalize (male advertisement vocalizations are dissimilar; Gerhardt, 2005). In total, I collected 23 amplexed males (along with their female mates) and 23 solo males (N = 46) taken at the same time (within 3 min) and location (within 5 m). In other words, males were collected in a pairwise fashion. This was done in order to control for potentially confounding factors such as time of night, temperature in pond, *et cetera*. Immediately upon collection, males were quickly (<5 min) moved to a processing station at the edge of the wetland. Here I recorded the clock time and measured the frog's body temperature prior to handling using an infrared digital thermometer (Fluke 62 Max + IR thermometer, Everett, WA; accuracy: ± 1.0 C). Then I collected whole blood via cardiac puncture—a technique that I have used successfully in gray treefrogs without adverse health effects (Baugh et al., 2021; Baugh et al., 2019; Gall et al., 2019). Briefly, I rapidly (<3 min) collected blood (ca. 50 μ L) using a 30-gauge insulin syringe (BD Micro-fine U-100, 0.3 mL) pre-rinsed with heparin. Using this technique in *Hyla versicolor* previously, Bastien et al. (2018) demonstrated that this method accurately captures plasma CORT concentrations without any elevation due to handling. Whole blood was stored at 4° C for 1–4 h and then centrifuged (7500 RPM for 10 min; Eppendorf 5418 at 8° C). The plasma fraction was collected and stored at –20° C for three weeks and then shipped on dry ice to Swarthmore College where samples were stored at –80° C for four months until assayed. Immediately after blood collection, I measured each frog's body mass (to the nearest 0.01 g) and two estimates of body length (to the nearest 0.01 mm): (1) snout-vent length (SVL), and (2) tibia-fibula length (TFL). The first 12 frogs sampled were not measured for mass and length (N = 34 with these biometrics). Frogs were returned to their site of collection at the end of each sampling period.

2.2. Steroid extraction and reconstitution

I have previously validated all the hormone methods in this species, including recovery determination, parallelism, and optimal dilution for these three steroids (see Gall et al., 2019). Here I conducted new optimal dilution validations for field-caught males. Our validations indicated that CORT and E₂ had optimal dilutions at 1:10 and testosterone at 1:80. I used 13–25 μ L of plasma from each male (one plasma sample was lost due to coagulation) which allowed us to measure CORT and T in 45 males (23 solo, 22 amplexed) with enough remaining plasma to measure E₂ in 35 males (20 solo, 15 amplexed). I used a liquid diethyl ether double extraction method that has proven effective for small volumes of plasma with high recoveries and low recovery variance among samples (see Baugh et al., 2012), including in frogs (Baugh et al., 2018; Bastien et al., 2018; Baugh et al., 2021; Gall et al., 2019). Plasma samples were micro-vortexed prior to subsampling and then added to borosilicate vials. Next, 200 μ L of ultrapure water was added to each vial in order to increase the aqueous volume for ease of decanting. I then added 2 mL of diethyl-ether to each vial and thoroughly vortexed and then froze the

aqueous layer on a slurry of dry ice and methanol. The organic layer was decanted to a new borosilicate vial and the aqueous layer was allowed to thaw; this extraction process was repeated a second time for each sample. The extracts were then dried under a stream of N₂ gas in an Evap-o-Rac (Cole-Parmer) at 37° C, resuspended in assay buffer (supplied by kit) at a 1:10 dilution and allowed to reconstitute for 12 h at 4° C.

2.3. Enzyme immunoassays

I estimated steroid concentrations using commercial EIA kits (DetectX® kits, Arbor Assays) for corticosterone (Cat. No. K014, Donkey anti-Sheep IgG), 17β-estradiol (Cat. No. KB30, Donkey anti-Sheep IgG), and testosterone (Cat. No. K032, Goat anti-Rabbit IgG). Reconstituted samples and kit reagents were allowed to reach room temperature prior to use and samples were vortexed prior to plating. Samples were randomly assigned to wells and assayed in duplicate (CORT, T) or as single wells (E₂) along with blanks, standard curve standards and pooled samples for use as internal standards in the calculation of intra- and inter-assay coefficient of variation (CV: [(SD/mean) * 100 %]. Samples were assayed following manufacturer instructions. I first subsampled for CORT and E₂, then further diluted samples to a final dilution of 1:80 for the measurement of T. Briefly, 50 μL of each sample or standard (or 100 μL for E₂) were plated into wells along with conjugate and antibody. Plates were then placed on an orbital shaker (500 RPM) at room temperature for 1 h (CORT) or 2 h (E₂, T) and then washed four times with wash buffer (supplied by kit). Substrate was then added and the plate was incubated at room temperature for 30 min without shaking. The reaction was stopped and optical densities were read at 450 nm on a Versa_{max} microplate reader with SoftMax Pro software using a four-parameter curve fitting equation (Molecular Devices). Intra- and inter-assay coefficients of variation (CV) were estimated using an internal standard at the beginning and end of each plate. I accepted the average of duplicate wells for CORT and T (E₂ in singlet wells). The assays have detection limits and sensitivities, respectively, of 16.9 pg mL⁻¹ and 18.6 pg mL⁻¹ for CORT, 2.05 pg mL⁻¹ and 2.21 pg mL⁻¹ for E₂, and 9.92 pg mL⁻¹ and 30.6 pg mL⁻¹ for T. The cross-reactivity of the antiserum for each kit is as follows: CORT: 100 % for corticosterone, 12.3 % for desoxycorticosterone, 0.62 % for aldosterone, 0.38 % for cortisol; E₂: 100 % for E₂, 3.2 % for estrone sulfate, and 2.5 % for estrone; T: 100 % for T, 56.8 % for 5α-dihydrotestosterone (hereafter DHT), and 0.27 % for androstenedione. The high cross reactivity of the T antibody for DHT might indicate that our T estimates may better represent testosterone plus DHT (i.e. 'androgens'). However, two recent studies using high performance liquid chromatography-mass spectrometry showed that plasma DHT concentrations are undetectable in closely related eastern gray treefrog females (*Hyla versicolor*; Bastien et al., 2018) and female túngara frogs (*Physalaemus pustulosus*; Baugh et al., 2018). Therefore, I hereafter provisionally refer to these estimates as testosterone. The intra- and inter-assay CV, respectively, were 9.56 % and 1.9 % for CORT; 3.67 % for E₂ (single plate); and 9.50 % and 7.94 % for T.

2.4. Statistics

I used SPSS® (Version 28, IBM) for statistical analyses. All hormone concentrations were log₁₀-transformed to improve residual distributions prior to statistical analyses. Assumptions of normality, homogeneity and sphericity of error variances were evaluated. I used several inferential test statistics, including independent t-tests (two-tailed), univariate and multivariate general linear models, Pearson's correlations and discriminant function analyses (DFA).

3. Results

Amplexed males had higher concentrations of all three steroid hormones relative to solo males (CORT: +347 %; T: +60 %; and E₂: +43 %);

independent t-tests (a) CORT: $t_{43} = 5.12$, $p < 0.0001$; (b) T: $t_{43} = 3.45$, $p = 0.001$; (c) E₂: $t_{33} = 3.32$, $p = 0.002$ (Fig. 1). Hormone concentrations were more variable (CV) in amplexed compared to solo males for the gonadal hormones ($T_{\text{amplex}} = 0.49$; $T_{\text{solo}} = 0.33$; $E_{2,\text{amplex}} = 0.36$; $E_{2,\text{solo}} = 0.21$) and substantially less variable in the same comparison for CORT ($\text{CORT}_{\text{amplex}} = 0.72$; $\text{CORT}_{\text{solo}} = 1.77$). When variances in all three hormones were included together in a multivariate general linear model (fixed factor: amplexus status; dependent variables: CORT, T, E₂) the same group effect was yielded: CORT: $F_{1,33} = 13.71$, $p = 0.0007$; T: $F_{1,33} = 9.74$, $p = 0.004$; E₂: $F_{1,33} = 11.05$, $p = 0.002$. To model the effects with all potential (co)variances included, I used a full factorial generalized linear model (dependent variable: amplexus status; predictor variables: CORT, T, and E₂ and all the two-way and three-way interactions; binary distribution with logistic link function). None of the interaction terms were significant (all $p > 0.2$) and were subsequently removed from the model leaving a main effects model. The main effects model confirmed a significant omnibus test (Likelihood Ratio Chi-Square = 29.55, $df = 3$, $p < 0.00001$) with significant effects for CORT (Wald Chi-Square = 6.20, $p = 0.013$) and E₂ (Wald Chi-Square = 4.19, $p = 0.041$) but not T (Wald Chi-Square = 1.66, $p = 0.198$). Alternative models with binary distributions and probit link functions yielded the same findings.

In solo males, a Pearson's correlation between residual body mass (RBM; body mass divided by TFL) and CORT yielded a negative correlation ($r = -0.630$, $p = 0.007$, $N = 17$) whereas no correlation was observed in amplexed males ($r = -0.364$, $p = 0.15$, $N = 17$). The difference in the slopes of these correlations, however, was not statistically significant—to evaluate this I used a general linear model with CORT as the dependent variable, amplexus status as the fixed factor and RBM as the covariate, retaining both of the main effects and the interaction term. The omnibus test was significant ($F_{3,30} = 14.42$, $p < 0.000005$) with significant main effects of amplexus status ($F_{1,30} = 5.48$, $p = 0.026$) and RBM ($F_{1,30} = 12.646$, $p = 0.001$) but a non-significant interaction term ($F_{1,30} = 3.44$, $p = 0.07$). No relationships between RBM and T or E₂ were found.

Amplexed males did not differ significantly from solo males in any other recorded variable, including residual body mass, body mass, body length (SVL, TFL), time of night sampled or temperature (all $p > 0.10$), as expected given the pairwise collection method. Further, frog temperature was not correlated with collection time ($r = 0.22$, $p = 0.14$, $n = 46$). Frog temperature, however, was negatively correlated with T in solo males ($r = -0.71$, $p < 0.001$, $N = 23$) but not in amplexed males ($r = -0.19$, $p = 0.39$, $N = 22$). No significant correlations between temperature and CORT or E₂ were found (all $p > 0.14$).

3.1. Discriminant function analysis

A DFA using all three hormone predictors simultaneously ($N = 35$ males) correctly classified males to amplexed and solo groups in 82.9 % of cases (29 of 35 males). This classification was significant and yielded one canonical discriminant function (eigenvalue = 1.184; canonical correlation = 0.736; Wilks Lambda = 0.458; $\chi^2 = 24.6$, $df = 3$, $p < 0.0001$). The standardized canonical discriminant function coefficients indicated that CORT had the highest loading followed by E₂ (CORT = 0.84; E₂ = 0.60; T = 0.36). The accuracy of the DFA increased nominally (85.7 %) when using a stepwise analysis method. The log₁₀-transformed hormone concentrations used in the DFA met the assumptions of univariate normality (Kolmogorov Smirnov; $p > 0.2$ for all three distributions) and multivariate normality (Box's M = 10.46, $p = 0.15$).

3.2. Hormone-hormone correlations

Each of the three steroids was not significantly correlated within male pairships (e.g., high CORT levels in a solo male did not predict high CORT in the amplexed male collected at the same time and location; all $p > 0.3$; SM1-SM3). There were no between-steroid correlations when

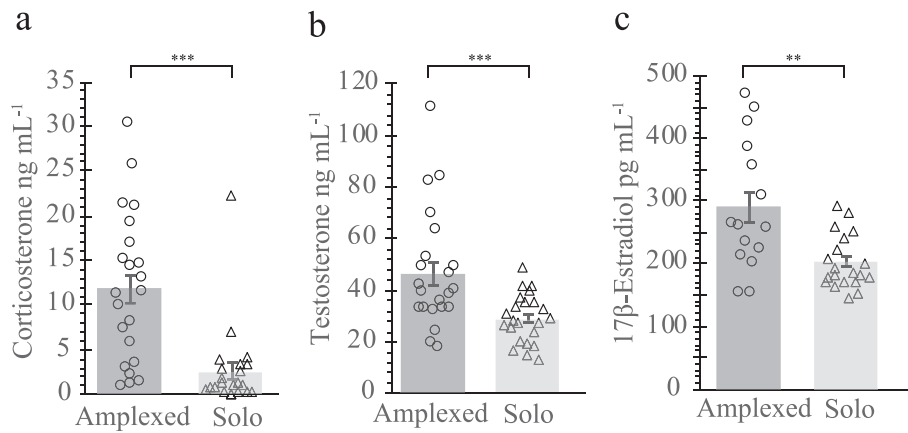


Fig. 1. Plasma hormone concentrations of male Cope's gray treefrogs sampled at natural breeding aggregations in either amplexed or non-amplexed (solo) states. Amplexed males had higher concentrations of all three steroid hormones compared to solo males. Hormones levels are depicted as untransformed, but were \log_{10} -transformed for analysis. Bars depict means with ± 1 standard error. Circles and triangle symbols represent individual amplexed and solo males, respectively. ** $p < 0.001$; *** $p < 0.0001$.

both amplexed and solo males were pooled, nor any between-steroid correlations within the solo male group (Fig. 2). However, in the amplexed males there was a positive correlation between T and E_2 ($r = 0.63$, $N = 15$, $p = 0.012$; Fig. 2b) and a borderline negative correlation between CORT and E_2 ($r = -0.51$, $N = 15$, $p = 0.05$; Fig. 2c).

3.3. Hormone-collection time correlations

Concentrations of CORT were higher in amplexed males collected and sampled earlier in the night relative to those collected later ($R^2 = 0.212$, $p = 0.031$), whereas no such relationship was evident in solo males ($R^2 = 0.0009$, $p = 0.89$; Fig. 3). A GLM (CORT as dependent variable) with main effects of amplexus status (fixed factor) and Clock Time (covariate) and interaction term (amplexus status * clock time) yielded a significant omnibus effect ($F_{3,41} = 10.33$, $p < 0.001$) with significant main effects of amplexus status ($F_{1,41} = 4.4$, $p = 0.042$) and clock time ($F_{1,41} = 4.59$, $p = 0.038$) and a borderline interaction term ($F_{1,41} = 3.99$, $p = 0.05$) suggesting that CORT declined across the night only in the amplexed males.

4. Discussion

Our findings indicate that reproductively active male Cope's gray treefrogs at the breeding aggregation can be categorized in a bimodal fashion with respect to their endocrine state as a function of their amplexus status. Amplexed males have higher and less variable CORT, higher T and E_2 , positively correlated levels of T and E_2 , negatively correlated levels of CORT and E_2 , and temporal lability in CORT wherein CORT is lower later in the night. Solo males, on the other hand, have relatively low concentrations of the three steroids and neither correlations among them nor temporal lability; lastly, T in solo males was negatively correlated with body temperature. In general, our findings are consistent with an associated reproductive pattern (Crews and Moore, 1986; reviewed in Woodley, 2011), wherein elevated gonadal hormones are associated with seasonal patterns of reproductive behavior. Here, however, that pattern is recapitulated in a condensed window of a few hours. Though reproductive behavior studies often focus on gonadal steroids (reviewed in Woodley, 2011), there is strong evidence across vertebrates that elevated GCs are common during breeding (reviewed in Romero, 2002). Moreover, elevated baseline GCs are coincident with the act of breeding in the present study species (Gall et al., 2019; Baugh et al., 2021) and others (reviewed in Leary and Baugh, 2020). Our results are also consistent with the narrower set of studies exploring how steroid hormone profiles vary as a function of mating activity in male amphibians (Townsend and Moger, 1987;

Orchinik et al., 1988; Lupo et al., 1993; Houck et al., 1996; Harvey et al., 1997; Reedy et al., 2014; O'Bryant and Wilczynski, 2010).

The elevated steroids in amplexed males suggests that the HPA/I and HPG axes are co-activated in amplexed males, potentially indicating a rapid integration of physiological parameters to support successful reproduction. Whether this (co)activation is the cause or consequence (or both) of the motor act of clasping (or associated sensory cues, including the mere exposure to a receptive female), will require further experimental work. Along these lines, there are at least three main hypotheses to consider.

Hypothesis 1. Correlated Sexual Selection: This hypothesis posits a unidirectional hormone-to-behavior causal relationship. Here, females select males by way of vocal characteristics that have an underlying correlation with hormone concentrations and hence the elevated steroid levels in amplexed males represent a kind of 'sampling bias' due to female choice (i.e. correlated selection). For example, if important male vocal parameters such as call effort (CE)—a measure of calling vigor that is the product of call duration (CD) and call rate (CR), which have been extensively shown to be important in female mate choice in this species and others (Klump and Gerhardt, 1987; Schwartz et al., 2001; Ward et al., 2013; Underhill and Höbel, 2017)—are positively correlated with gonadal and/or interrenal steroids, this could result in correlated sexual selection on the hormone concentrations, thereby yielding sexier males because (indirectly) they had higher steroids. A related study provides some support for this interpretation: males with higher E_2 and T called more vigorously (CR and pulse duty cycle). The effect sizes here, however, are too weak to adequately explain the hormonal divergence between solo and amplexed males observed in the present study; moreover, no correlations between CORT and vocal parameters have been observed in this species (Baugh, in prep.). Hence, there is currently weak, partial support for Hypothesis 1 for gonadal hormones and no support for interrenal hormones.

Hypothesis 2. Amplexus behavior drives hormones. This hypothesis (2a) posits a unidirectional behavior-to-hormone causal relationship. Here, the physical act of getting into amplexus with a female might stimulate both the HPI and HPG axes in males resulting in elevated steroids. A sub-hypothesis (2b) might posit the mere sensory detection of a reproductive female by a male, as opposed to or in addition to the physical act of amplexus, stimulates the HPG/I axes. This hypothesis assumes relatively rapid reactivity of both endocrine axes given that males are unlikely to be in amplexus more than a few hours at the most prior to collection and blood sampling. This assumption is supported for the HPA/I axis as it is well known for its rapid response to life history challenges, with extrinsically induced CORT concentrations becoming elevated within less than 3 min (Baugh et al., 2015; Baugh et al., 2017; Romero and Reed, 2013). Similarly, in male rats the opportunity to mate

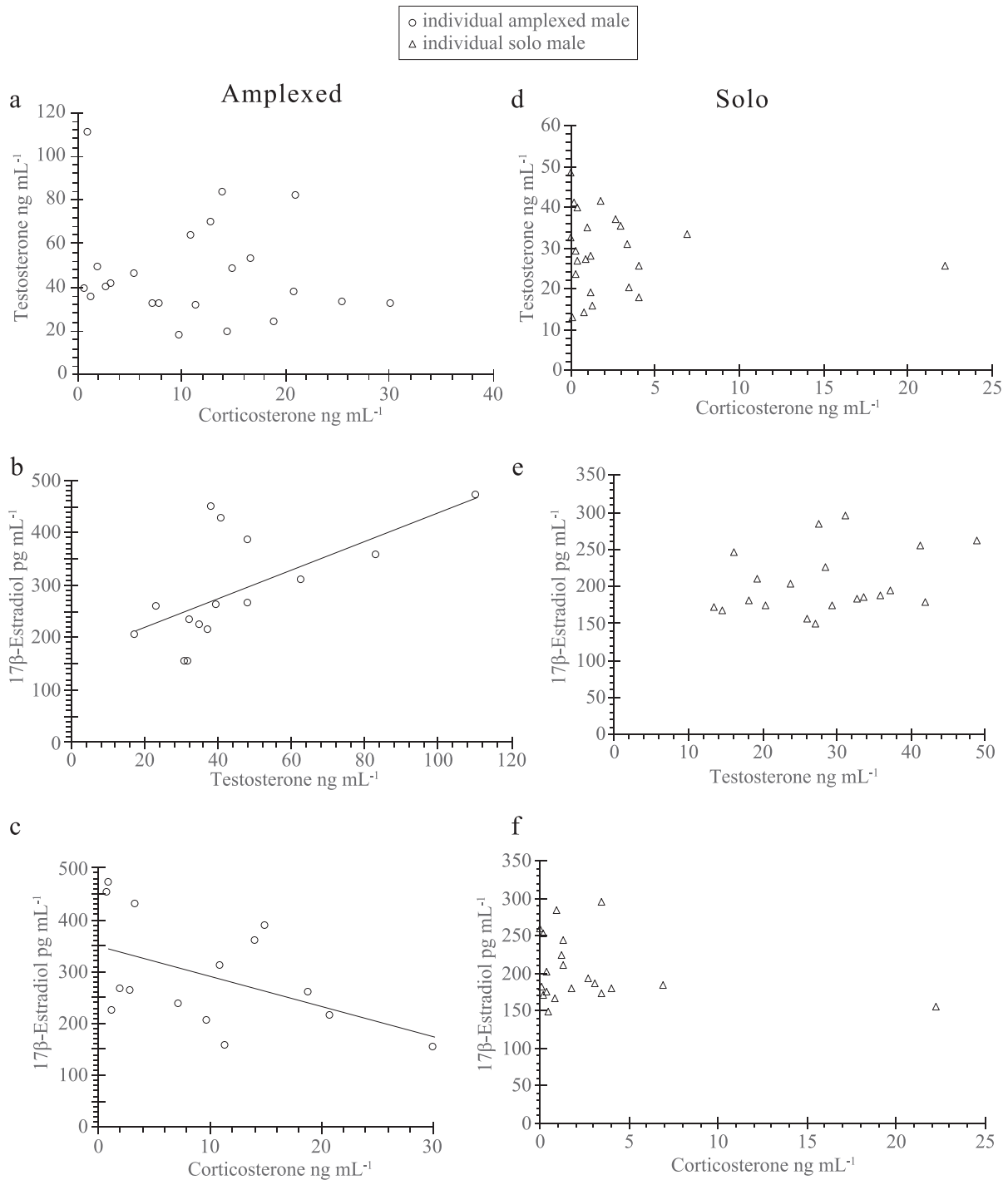


Fig. 2. Pearson's correlations among steroid hormones for males sampled in amplexus or non-amplex (solo). Hormones levels are depicted as untransformed, but were log₁₀-transformed for analysis. Significant correlations were only observed in amplexed males (b, c).

with a reproductively receptive female is known to induce a large spike in CORT (Buwalda et al., 2012).

Hypothesis 3. Effects of Correlated Selection are amplified and/or followed by hormone activation via amplexus. This hypothesis posits a specific bidirectional hormone-behavior relationship and therefore is not mutually exclusive of Hypotheses 1 and 2. Here, males with elevated gonadal steroids are more likely to be chosen by females due to their correlated vocal traits, and then the physical act of amplexus stimulates the HPG axis and potentially also further stimulates the HPG axis thereby amplifying the initial (pre-choice) differences. A sub-hypothesis (3b) could posit that merely the sensory

detection of a reproductive female, as opposed to or in addition to the physical act of amplexus, elevates and amplifies one or more hormones.

Testing these hypotheses will require experimental manipulation. For example, a solo male can be induced to perform amplexus by providing him with a receptive female from a pair collected in amplexus with her chosen mate removed (pers. obs.). This experimental manipulation would provide an improved opportunity to test whether the association observed in the present study—elevated steroids in amplexed males—are the cause or consequence of amplexus. However, such a design involves additional handling and holding in captivity,

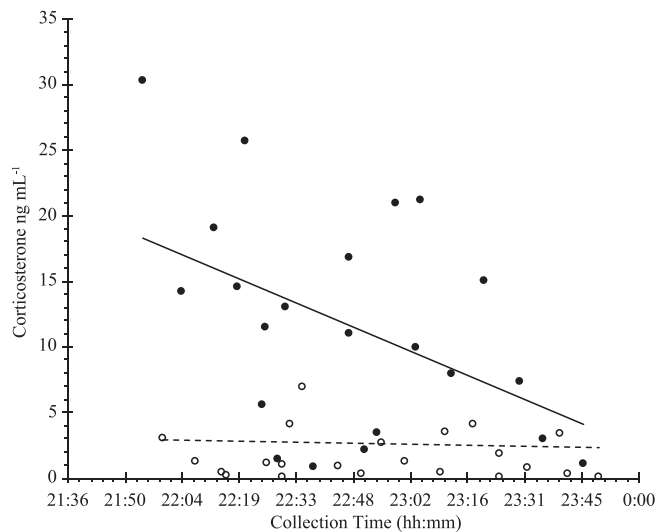


Fig. 3. Pearson's correlation for collection time of day and plasma CORT concentrations for males collected in amplexus (solid circles, solid line) and solo males (open circles, dotted line). The correlation was significant for amplexed males but not solo males. Hormones levels are depicted as untransformed, but were \log_{10} -transformed for analysis.

which might itself alter HPI activation. Moreover, an additional limitation arises for causal inference: induction of amplexus coincides with the termination of calling behavior. Hence, endocrine changes associated with amplexus might be driven by the metabolic shift towards supporting amplexus *per se*, the shift away from supporting vocal effort, or both. Because males cannot vocalize during amplexus, overcoming this challenge may involve methods that experimentally suppress calling from active males, coupled with experimental manipulation of amplexus status. Studies in newts, which do not vocalize, also demonstrate that amplexus is energetically costly (Coddington and Moore, 2003) and associated with elevated CORT (Reedy et al., 2014). However, because newts do not vocalize, the transition to amplexus involves a net increase in aerobic respiration whereas in gray treefrogs that transition likely involves a net decrease (McLister, 2003).

Because of the high energetic demands of vocalizing in male anurans (Ryan et al., 1983; Taigen and Wells, 1985), they often have elevated levels of CORT relative to non-vocal neighbors (Emerson, 2001). This is thought to arise because elevated CORT stimulates gluconeogenesis (Exton et al., 2013), though this likely depends on life history tactics (Crocker-Buta and Leary, 2018a, 2018b) and body condition (Joshi et al., 2018; Walkowski et al., 2019). In the present study, however, amplexed males had higher (and less variable) CORT than their actively vocalizing neighbors. There are multiple possible interpretations for these findings. First, elevated CORT in amplexed males may reflect a longer tenure (i.e. higher effort) vocalizing at the lek that night. If part of what makes a male successful in attracting a female mate is attendance at the lek (Ryan, 1985), then I might predict more vocally active males (calling more intensively, or for a longer duration, or both) to both have higher probabilities of attracting a mate and to have higher CORT to sustain those earlier efforts. However, given that solo males were actively and vigorously calling immediately prior to sampling, it seems unlikely that elevated CORT in amplexed males is a referent for higher vocal activity. Second, elevated CORT in amplexed males might suggest that the relative energetic demands of amplexus exceed those of vocal effort. Hence, elevated CORT might enable males to shift from a high energetic demand during vocal advertisement to an even higher one during amplexus. This also seems unlikely given that vocal effort demands higher absolute aerobic respiration than amplexus in the closely related eastern gray treefrog (McLister, 2003). Likewise, there was a negative correlation between residual body mass and CORT in solo

males only, suggesting that solo males with elevated CORT were potentially in a negative energy balance condition, which could reflect more recent calling activity (Walkowski et al., 2019). However, net metabolic increases are likely not the only governing variable for elevated CORT. For example, the estimated metabolic demands to support amplexus are higher per gram of muscle compared to that of vocalizing (McLister, 2003), and this might require shifts in HPI activity. Lastly, the substantially less variable CORT concentrations observed in amplexed males might result from variability in the vocal efforts of solo males (Runkle et al., 1994), with highly vocal males exhibiting relatively high CORT and vice versa. In contrast, amplexed males are engaged in a presumably similar motor and thus energetic regime which may explain their less variable CORT levels.

Solo and amplexed male frogs differed in more than just plasma hormone concentrations. Amplexed males exhibited positively correlated T and E₂ levels, which may indicate thresholding effects of HPG axis activation potentially due to elevated aromatase activity converting some of the abundant plasma T into E₂ (Chakraborty and Burmeister, 2009). Further, in amplexed males E₂ was negatively correlated with CORT. Because glucocorticoids are known modulators of aromatase activity (McTernan et al., 2002; Brooks et al., 2012), this negative correlation might indicate that high CORT suppresses aromatase activity in the testes (and interrenals). Lastly, amplexed males collected later in the night had lower CORT levels, whereas no such correlation was observed in solo males. While, circadian rhythms in CORT secretion are known in amphibians (Titon et al., 2021), the fact this correlation was only observed in amplexed males suggests this effect is not driven by diel patterns. Because the act of amplexus is coincident with the cessation of vocal activity, and because vocal activity is energetically demanding (Emerson and Hess, 2001; Wells and Schwartz, 2007; Exton et al., 2013), the lower CORT in amplexed males late at night might reflect a longer interval of non-calling (or a longer interval since male-male interactions) and the declining CORT that accompanies it. This assumes that males collected later in the night engaged in amplexus at approximately the same time of night as males collected earlier. Instead, males collected later might have only recently amplexed a female, and this apparent decline might reflect the fact that their CORT elevation has not yet reached peak levels. Alternatively, the energetic costs of maintaining amplexus may wane over time, resulting in diminished CORT secretion. Lastly, the mere presence of a reproductive female is known to stimulate the HPA axis in laboratory rats (Buwalda et al., 2012), and therefore it is possible that the super-elevated CORT levels in amplexed males earlier in the night reflect that initial socially-induced spike, which attenuates over subsequent hours. Because vocal activity and male-male interactions are known to influence plasma CORT and T (de Assis et al., 2012; but see Joshi et al., 2017), future efforts will need to experimentally control for variation in behavior preceding amplexus.

Hence, experimental research is needed to understand how variable metabolic demands across tissue types are differentially regulated by glucocorticoids and potential non-linearities in those relationships. Because CORT was the steroid that experienced the highest difference in amplexed compared to solo males (+347%), this may reflect a major metabolic transition. This very elevated CORT combined with the fact that the HPA/I axis tends to react faster (ca. 3 min) than the HPG axis (Romero and Reed, 2005; Baugh et al., 2013, 2017), might suggest that coactivation might be governed via initial secretory increases in CORT, which may then interact with the HPG axis (Wingfield and Sapolsky, 2003; Toufexis et al., 2014). Because amplexus is typically coincident with the cessation of calling and male-male interactions in male anurans, the frequency and intensity of these behaviors prior to amplexus deserve study, as they are relevant for broader concepts in the directionality of hormone-behavior relationships, including proposed the "Energetics-Hormone Vocalization" model (Emerson, 2001) and the Challenge Hypothesis (Goymann et al., 2019). Finally, given the diversity of calling strategies (Crocker-Buta and Leary, 2018a, 2018b), agonistic behaviors (Wells, 2007; de Assis et al., 2012), and seasonality

among anurans (Runkle et al., 1994), a broader comparative treatment is warranted.

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Ethics

Animal collections were made under Special Permits 23,543 and 28,347 from the State of Minnesota Department of Natural Resources. This study was approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Protocol #2001-37746A).

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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