Testosterone and Corticosterone Profiles and Body Condition of Calling and Non-calling *Lithobates grylio*

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Anuran vocalization is one of the most energetically expensive behaviors recorded in vertebrates. To sustain this behavior, circulating hormones in male frogs must act to promote calling behavior while mobilizing energy reserves. We hypothesize that this is accomplished through the actions of testosterone (T) and corticosterone (CORT). T is thought to elicit calling, while CORT mobilizes energy reserves. Eventually, high CORT concentrations and low energy reserves suppress calling behavior. We evaluated this hypothesis in light of the reproductive ecology of the Pig Frog, *Lithobates grylio*. Plasma samples from calling and non-calling males were collected during four months of the reproductive season. Calling males were shown to have significantly higher concentrations of circulating T and high body conditions. Conversely, non-callers exhibited elevated CORT and low body conditions. There was no correlation between the two hormones, but circulating T concentrations did decrease over the season. Lastly, we found that CORT is positively correlated with circulating glucose (in non-callers); this finding is supported by CORT's known role in gluconeogenesis. Taken together, these data indicate that circulating hormones and available energy reserves likely influence calling behavior in *L. grylio*.

ANURAN vocalization is one of the most energetically expensive activities recorded in ectotherms, and much research exists on the physiological mechanisms that elicit, maintain, or suppress this behavior (Bucher et al., 1981; Taigen et al., 1985; Pough et al., 1992). One hypothesis on these mechanisms is the Energetics-Hormone Vocalization (EHV) model, which proposes that endocrine response during the anuran reproductive season is driven by the high energetic costs of calling (Emerson, 2001). This model proposes that calling behavior is elicited by the influence of androgens, is energetically maintained by an increase in glucocorticoids, and is subsequently suppressed by high concentrations of glucocorticoids (Emerson, 2001; Emerson and Hess, 2001). Past research has shown that testosterone (T) promotes calling, which causes an increase in energy consumption by the muscles responsible for vocalization (Leary, 2009; Zornik and Kelley, 2011). Conversely, corticosterone (CORT) is thought to suppress the effects of T and promote gluconeogenesis, which mobilizes energy reserves (Holberton et al., 1996; Wingfield et al., 1998). Thus, the EHV model suggests that T and CORT are inherently linked under the EHV model through the persistence of this energetically expensive behavior.

Studies on the relationship among androgens, glucocorticoids, and calling behavior have focused on the hormonal differences between calling and non-calling males (Brzoska and Obert, 1980; Licht et al., 1983; Mendonça et al., 1985; Leary et al., 2006, 2015; Gramapurohit and Radder, 2013; Madelaire and Gomes, 2016; Titon et al., 2016; Joshi et al., 2017). The EHV model predicts that calling males have high concentrations of circulating T, and as calling behavior persists, CORT concentration rises (Emerson, 2001; Emerson and Hess, 2001). For at least some frog species, when energy reserves are depleted, CORT reaches a critical concentration and acts to inhibit calling behavior (Wilczynski et al., 2005; Leary et al., 2015). CORT is responsible for the mobilization of these energy reserves during reproduction, which explains why an increase in CORT is shown to be correlated with low body condition (Leary et al., 2015; Titon et al., 2016). Conversely, high body condition and large fat reserves are positively correlated with calling behavior (Madelaire and Gomes, 2016; Joshi et al., 2017).

Differences in hormone concentration between the calling and non-calling ethotypes vary interspecifically due to the different energetic requirements of the reproductive behaviors involved (Wingfield et al., 1990). In species with an explosive breeding strategy, like *Anaxyrus woodhousii* and *A. cognatus,* calling activity persists only for one week and males do not forage during this time (Krupa, 1989; Sullivan, 1989). For these species, CORT injections have no effect on circulating T concentrations, but CORT injections do induce non-calling status (Leary et al., 2006). In contrast, for species with a prolonged breeding strategy, circulating T concentrations are negatively correlated with CORT (Mendonça et al., 1985; Marler and Ryan, 1996; Leary et al., 2015). This suggests that the potential negative effect of CORT on T will only be found in species that maintain calling behavior for months out of the year. However, it is unclear whether CORT has a direct effect on T or if suppression of androgen-mediated behaviors is due to CORT's actions within a different physiological pathway (Marler and Ryan, 1996; Emerson and Hess, 2001; Leary et al., 2004; Wilczynski et al., 2005; Leary, 2009; Leary and Harris, 2013).

Androgen and glucocorticoid concentrations are also influenced by temporal and social factors. Androgen concentration fluctuates during the breeding season, where T spikes during the onset of reproductive season (O'Bryant and Wilczynski, 2010). There is a marked drop in circulating androgens as the season progresses (Licht et al., 1983; O'Bryant and Wilczynski, 2010; de Assis et al., 2012). Social stimuli, such as exposure to conspecific calls, are positively correlated with circulating androgens, testes weight, and calling behavior (Brzoska and Obert, 1980; Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001; Leary, 2009). CORT is also known to peak during the reproductive season, which allows energy to be allocated to reproductive behaviors (Romero, 2002; Berner et al., 2013).

Lithobates grylio is a large bodied and fully aquatic anuran endemic to the swamps and marshes of the southeastern United States (Wright, 1932). During the prolonged reproductive season (March–September), males migrate to deeper

water to advertise (Ligas, 1960; Wells, 1977; Lamb, 1984). Calling males exhibit a distinct behavior by floating on top of the water with their throat and forelimbs extended anteriorly (Lamb, 1984). During this reproductively active period, the males are thought to defend call sites and decrease foraging activity (Lamb, 1984; Ugarte et al., 2007). Understandably, energy expenditure is high in males of *L. grylio* because they call for long periods of time, defend a territory, and, concurrently, eat less. This combination of behaviors raises questions about how energy is allocated during the reproductive season. In accordance with the EHV model, the physiologies of calling and non-calling *L. grylio* should be distinct with regard to glucocorticoids and androgens. By measuring CORT and T concentrations in calling and non-calling *L. grylio,* we tested the hypothesis that physiological characters that regulate calling behavior would be related to reproductive ecology. We predicted: (1) that calling males would have higher body conditions; (2) that T concentration would be higher in calling males; (3) that CORT concentration would be highest in non-calling males; (4) that since *L. grylio* is a prolonged caller, T would be negatively correlated with CORT; (5) that T would be positively correlated with ambient call rates; (6) that over the calling season, T concentrations and body condition would decrease while CORT concentrations increase; and (7) that since CORT is associated with the mobilization of energy reserves, it would be positively correlated with circulating glucose concentration.

MATERIALS AND METHODS

Fieldwork.—Fieldwork took place from June through September 2014 in Doubloon Branch Bayou, a freshwater marsh system located in St. Tammany Parish, Louisiana. All fieldwork was conducted between 2300 and 0100 hours. A Sony ICRecorder equipped with a mixer and two microphones was placed 1 m above the surface of the water to record nightly chorus activity. Water temperature was measured using an aquatic, digital thermometer. Air temperature and barometric pressure were monitored through an AcuRite wireless weather station.

The field site was navigated by paddle in a flatboat (3 m in length) and consisted of open water habitat containing islands of floating vegetation. The dominant flora of the vegetated microhabitats was Alligator Weed (*Alternanthera philoxeroides*) and Swamp Primrose (*Ludwigea peploidess*).

A spotlight was used to make behavioral observations prior to approaching via boat and capturing frogs. Upon spotlighting, two observers observed one minute of behavior from the boat. Reproductively mature males were identified by enlarged tympanic membranes and deemed "calling" or "non-calling" (Dundee, 1974). The "shy," fully aquatic nature of *L. grylio* made extended focal observations impossible (Wright, 1932). Males were readily identified as calling if males were producing vocalizations and exhibited inflated abdomens and high body position (Lamb, 1984). The approach and capture upon focal observation also acted to reduce the potential of a visually induced stress response (Narayan et al., 2013).

Frogs were captured by hand, and 0.5 mL of whole blood was drawn immediately via cardiac puncture with a heparinized, 26 gauge, ¾-inch needle, using methods established in previous studies (Licht et al., 1983; Mendonça et al., 1985; Leary et al., 2004; Leary and Harris, 2013; Madelaire and Gomes, 2016). Blood draw occurred aboard the boat, and

whole blood was stored in 1.5 mL non-heparinized centrifuge tubes placed on ice. If blood samples were not acquired within five minutes from the onset of focal observation, the frogs were released and data were not recorded. Visual exposure to a predator is enough to stimulate the HPI axis; therefore, our study used five minutes from the time of spotlighting as our cutoff time for plasma sample collection (Narayan et al., 2013). Each frog was marked by a unique toe clip pattern, which allowed individual identification in subsequent recaptures. Specimens were measured with calipers and weighed with a hanging scale. These morphological data were later used to make inferences about the body condition of the frogs. Here, we define body condition as phenotypic variation that can be used as a proxy for the total amount of energy reserves available to an animal (Schulte-Hostedde et al., 2011; Labocha et al., 2014).

After the night's fieldwork, blood samples were centrifuged at 10,000 rpm for ten minutes. Plasma samples were transferred to fresh 0.5 mL non-heparinized centrifuge tubes and stored at –20°C overnight. Samples were then transported on ice to Southeastern Louisiana University and stored at –80°C until analysis. Samples were taken from 36 individuals over the reproductive season. Five individuals were recaptured twice, one individual was recaptured three times, and another individual was recaptured four times yielding a total of 46 plasma samples. Days between resampling the same individuals ranged from 8–94 days.

Call analysis.—The call of *L. grylio* is a series of low frequency "grunts," which makes calculating call rate by ear possible (Wright, 1932). Call rates of the nightly chorus were counted in the playback of field audio recordings spanning 2359 to 0001 h. Individual frogs were not identified in these recordings; instead, the recordings were used to evaluate the ambient, nightly call rates. In previous work, ambient call rates were shown to influence circulating androgens in calling and non-calling frogs; therefore, our ambient call measurement was later evaluated with respect to circulating T within individual frogs (Burmeister and Wilczynski, 2000; Wilczynski and Chu, 2001; Gall and Wilczynski, 2015).

Hormone assays.—Commercial enzyme linked immunosorbent assay (ELISA) kits were used to measure T (ENZO Life Sciences, Inc. Farmingdale, NY) and CORT concentrations (Cayman Chemical Company Ann Arbor, MI) in the plasma samples. Manufacturer reported cross-reactivity for the T antibody was T (100%), 19-hydroxytestosterone (14.6%), Androstendione (7.20%), Dehydroepiandrosterone (0.72%), Estradiol (0.40%), and <0.001%: Dihydrotestosterone, Estriol, Aldosterone, Corticosterone, Cortisol, Cortisone, Estrone, Progesterone, and Pregenolone. CORT antibody cross-reactivity was CORT (100%), 11-Dehydrocorticosterone (11%), 11-Deoxycorticosterone (7%), Progesterone (0.31%), Cortisol (0.17%), Aldosterone (0.06%), Testosterone (0.03%), Pregnenolone (0.02%), 5- αDHT (0.01%), and <0.01%: Androstenedione Cortisone DHEA and DHEA-S. A parallelism test using a serial dilution of pooled blood plasma was completed to validate that the antibody specifically bound to the steroid hormones in *L. grylio*. Serial dilution of the kits' hormone standards and serial dilution of plasma samples exhibited parallel absorbance curves, indicating that the hormones in the plasma of *L. grylio* bound to the antibodies in a concentration-dependent manner. The plasma volume used for analysis was dependent upon the type of hormone measured. CORT extraction volume was 50 µl and T was
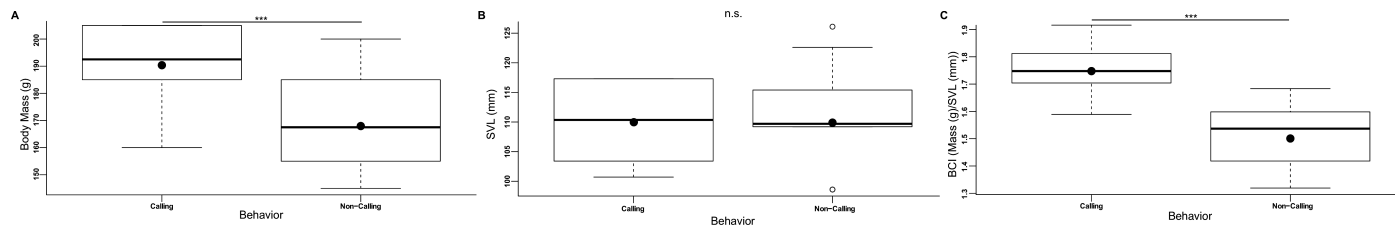
Fig. 1. Boxplots illustrating average (A) body mass, (B) snout–vent length (SVL), and (C) body condition index (BCI = mass/SVL) for calling ($n = 11$) and non-calling ($n = 14$) males. Calling males had significantly higher body masses (A; $t_{23} = 3.725$; $P = .001$) and body condition indices (C; $t_{23} = 5.599$; $P < 0.001$) than non-callers. SVL did not significantly differ (n.s.) between the two ethotypes (B; $t_{23} = 0.577$; $P = 0.570$). Box size is based on the range of data points falling between the 2nd and 3rd quartiles. Mean is represented by the filled circle and median by the bold line. Open circles denote potential outliers that were not omitted from statistical analysis.

between 40–30 μl; hormone concentrations were corrected for this difference in plasma volumes. Hormones were extracted using a double ether extraction protocol to maximize extraction efficiency, where the ether was evaporated at 37°C under a stream of N_2 gas. EIA buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) was used to reconstitute the samples. The assays were then performed according to the manufacturer's instructions. Samples were run in triplicate. Plates were read on a Power Wave HT Microplate Spectrophotometer at 405 nm (T) or 415 nm (CORT). A plasma pool with known hormone concentrations was plated in nine wells of each plate in order to assess intra- and inter-assay variance. Intra-assay variation for T was 7.28%, and inter-assay (between two plates) variation was 9.32%. Intra-assay variation for CORT was 4.83%, and inter-assay (between three plates) variation was 5.28%.

Quantifying glucose concentration.—A TRUETrack® glucometer was used to measure glucose concentration in the plasma that had been stored at –80°C by placing 3 μl of blood plasma onto the test strips in accordance with manufacturer guidelines. Glucose samples were tested in triplicate.

Statistical analysis.—All statistical analyses were completed using SigmaPlot 13 and alpha for significance was $P \leq 0.05$. All *post hoc* analyses were completed using Tukey's HSD tests. Separate t-tests were used to examine the relationship between morphometric data (snout–vent length [SVL], body mass (g), and body condition index [BCI]) and ethotype. BCI was calculated as the ratio of body mass to SVL (mm). This calculation is commonly used as a means to non-invasively assess individual condition and is used as a proxy for energetic reserves (Labocha et al., 2014). Additionally, to verify body condition and account for allometry, an ordinary least squares (OLS) regression between SVL and body mass was conducted; the residuals from the regression line were calculated for both calling and non-calling males. A t-test was used to compare the residuals of the ethotypes. A one-way ANOVA was conducted to test the relationship between month and BCI. A backward stepwise multiple regression was used as an exploratory analysis on the effect of air temperature, water temperature, and barometric pressure on call rates of *L. grylio*. A linear regression was used to determine the relationship between air temperature and call rates.

The coefficient of variance (CV) between replicates was assessed for T, CORT, and glucose measurements. Our protocol requires %CV be less than 5% between samples, 10% within a plate, and 15% between plates. Samples were only included in analyses if they had less than 5% of variation between replicates; 10 T, 8 CORT, and 17 glucose measurements were not included in analysis due to high %CV. Outliers were removed based on studentized-t values of ±3. Kolmogorov-Smirnov (KS) and Levene's tests were used to test statistical assumptions.

Average concentrations of T, CORT, and glucose were compared for callers and non-callers using separate two sample t-tests. The natural logs of T and glucose concentration were used to meet the assumption of normality. One-way ANOVAs were used to test the association between month and T across both behavioral types. A non-parametric ranking was employed to meet statistical assumptions. The square root of CORT was used to meet assumptions of normality and homogeneous variance.

Linear regressions were used to assess the relationship between CORT and glucose for callers and non-callers, where one outlier was excluded from analysis based on a studentized-t of +3. Another linear regression tested for correlation between CORT and T within each ethotype, where a natural log transformation of both hormone concentrations was necessary to meet statistical assumptions. Call rate (per night) and circulating T concentration (per individual) was assessed with linear regression. Linear regressions were also used to test for a relationship between BCI and circulating hormones. In all linear regressions, one-sample KS tests and scatterplots of the residuals were used to test the statistical assumptions.

RESULTS

Males exhibited distinctive posturing behavior which is consistent with Lamb's (1984) observations. Calling males floated high in the water, while non-calling males exhibited a low body position with only the top of their snout and eyes exposed above the water. Body mass was only obtained from 11 calling males and 14 non-calling males over the season; therefore, morphometric statistics only included these individuals. There was no significant relationship between SVL and behavior ($t_{23} = 0.577$; $P = 0.570$; Fig. 1B), yet calling males had significantly higher body masses ($t_{23} = 3.725$; $P = 0.001$; Fig. 1A) and BCIs ($t_{23} = 5.599$; $P < 0.001$; Fig. 1C) than non-callers. There was a significant positive linear relationship between body mass and SVL ($R^2 = 0.21$; $\hat{Y}_i = 37.64 + 1.26X_i$; $P = 0.02$; Fig. 2A). Calling males had significantly higher residuals from this OLS regression line ($t_{23} = 5.22$; $P < 0.001$; Fig. 2B). There was no difference in BCI between months ($F_{2,21} = 1.157$; $P = 0.333$). There was no significant correlation between BCI and CORT ($R^2 = 0.077$; $P = 0.237$) or BCI and T ($R^2 = 0.127$; $P = 0.105$). There were two instances of males switching from calling in the early season (June) to non-calling later in the season (August). There was
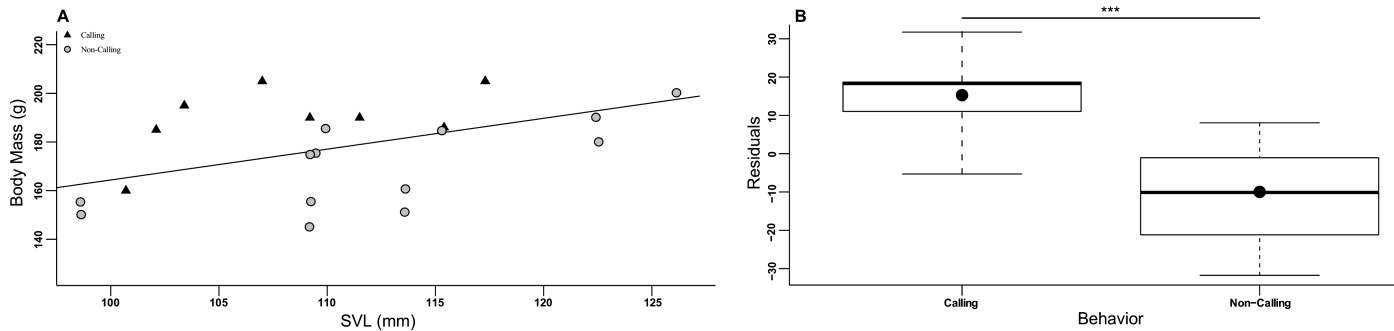
Fig. 2. (A) A linear regression of SVL and body mass suggests a positive linear allometric relationship between the variables ($R^2 = 0.21$; $\hat{Y}_i = 37.64 + 1.26X_i$; $P = 0.02$). (B) A boxplot of the residuals from Figure 2A for the ethotypes indicates calling males have significantly higher body masses than what is predicted by the SVL and body mass regression line ($t_{23} = 5.599$; $P < 0.001$). Box size is based on the range of data points falling between the 2nd and 3rd quartiles. Mean is represented by the filled circle and median by the bold line.

only one instance where an early season non-caller was observed to be calling at a later recapture date. Unfortunately, due to low sample size and variation in the hormone assays, physiological correlations could not be tested on recapture data.

Average circulating T concentration was significantly higher in calling males ($t_{34} = 2.987$; $P < 0.01$; Fig. 3A). Calling males were only captured in the early collecting season (June and July). The entirety of the late season (August and September) sample was comprised of non-calling males. Average T concentration across both groups showed a significant decrease from early to late season ($F_{3,31} = 16.187$; $P < 0.001$; Fig. 4). Audio recording taken in the field allowed for ambient call analysis for 16 nights across the sampling season. Circulating T showed a positive correlation with ambient call rates when calling and non-calling males were considered together ($R^2 = 0.285$; $\hat{Y}_i = 3895.38 + 31.08X_i$; $P < 0.01$; Fig. 5). The only variable that was correlated with call rates of *L. grylio* was air temperature. As air temperature increased and peaked in September, call rates significantly dropped ($R^2 = 0.52$; $P < 0.05$).

Average CORT concentration was higher in non-calling males than in calling males ($t_{36} = 2.153$; $P = 0.0381$; Fig. 3B). There was no significant difference in CORT concentration across months ($F_{3,34} = 0.234$; $P = 0.872$). No significant correlations between T and CORT concentrations were found for calling ($R^2 = 0.04$; $P = 0.38$; Fig. 6) or non-calling ($R^2 = 0.09$; $P = 0.77$; Fig. 6) males.

Glucose measurements were obtained for only 19 individuals (10 callers; 9 non-callers) due to high %CV and plasma volume availability. Circulating glucose was not significantly

different between the ethotypes ($t_{17} = 1.565$; $P = 0.136$). However, there was a significant positive relationship between circulating CORT and glucose concentrations in non-calling males ($R^2 = 0.820$; $\hat{Y}_i = 33.731 + 0.098X_i$; $P = 0.013$; Fig. 7). The same was not found for calling males ($R^2 = 0.149$; $P = 0.305$; Fig. 7).

DISCUSSION

The EHV model predicts that calling behavior is energetically constrained due to the depletion of energy reserves during calling (Emerson, 2001). The data reported herein support this hypothesis and show that the body condition of calling *L. grylio* was significantly higher than that of non-callers. Our study used BCI and OLS residuals as proxies for energy storage, both of which are shown to be correlated to fat stores in other species (Ardia, 2005; Schulte-Hostedde et al., 2011; Labocha et al., 2014). Previous work indicated that as energy reserves are used, the BCI of calling males begins to decrease (Sullivan and Kwiatkowski, 2007). These energy reserves are important in maintaining chorusing behavior, and hence non-calling males typically have low body condition (Murphy, 1994; Wells, 2001; Leary and Harris, 2013). Thus, it is likely that *L. grylio* rely on mobilization of energy stores to sustain prolonged calling behavior over the reproductive season.

T concentration was higher in calling males in the present study. Testosterone showed a marked drop throughout the season, with the early season males having a significantly higher concentration than late season males. This finding was consistent with previous work completed on a wide
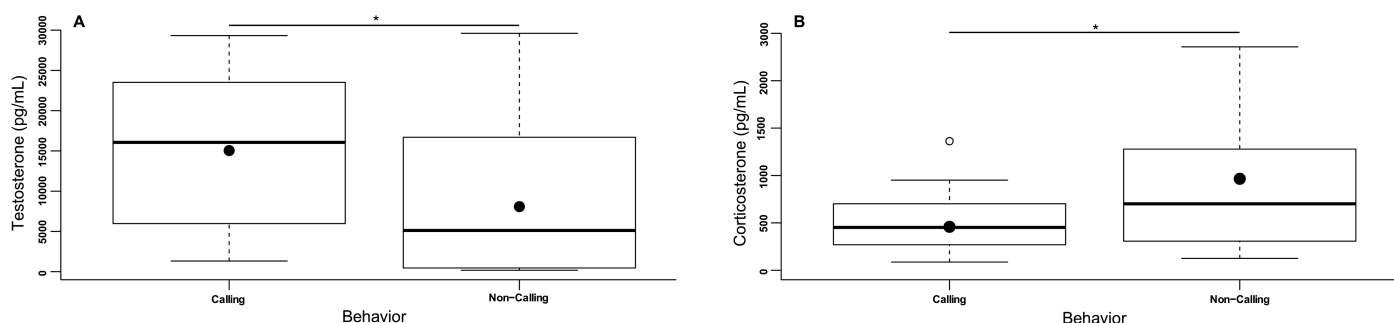

Fig. 3. (A) Average circulating T in calling ($n = 20$) and non-calling males ($n = 16$). The numbers on the bars indicate sample size. T was significantly higher in callers ($t_{34} = 2.987$; $P < 0.01$). (B) Average circulating CORT in calling ($n = 19$) and non-calling ($n = 17$) males, where CORT was elevated in non-callers ($t_{36} = 2.153$; $P = 0.0381$). Box size is based on the range of data points falling between the 2nd and 3rd quartiles. Mean is represented by the filled circle and median by the bold line. Open circles denote potential outliers that were not omitted from statistical analysis.
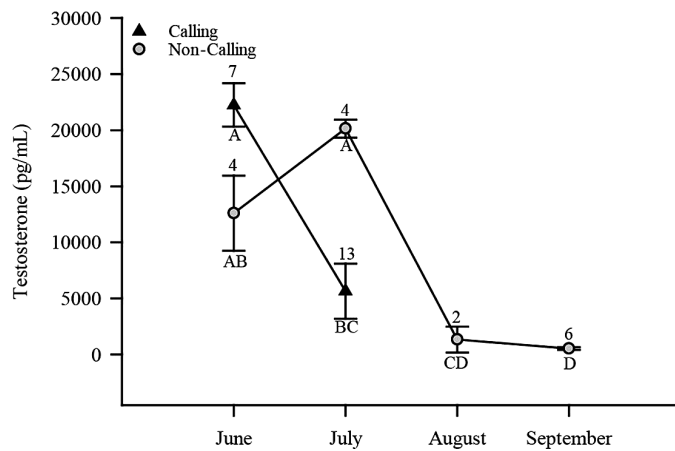
Fig. 4. Average T concentration for calling and non-calling males of *L. grylio* across months, where like letters indicate non-significant differences in concentrations and numbers indicate sample size. A significant seasonal decrease was observed in circulating T ($F_{3,31} = 16.187$; $P < 0.001$).

variety of vertebrate taxa (Goymann et al., 2007), including anurans (de Assis et al., 2012). Because males with relatively low circulating androgen concentration were still capable of vocalizing, the role T plays in eliciting calling behavior in *L. grylio* remains unclear. Previous work showed that the influence of androgens on calling behavior seemed to be species specific, and comparisons of T concentrations in other species have also produced variable results (Mendonça et al., 1985; Marler and Ryan, 1996; Wilczynski et al., 2005).

In anurans, social stimuli such as exposure to conspecific calls can cause an increase in circulating androgens, testes weight, and sensitivity of peripheral structures associated with signal reception (Brzoska and Obert, 1980; O'Bryant and Wilczynski, 2010; Gall and Wilczynski, 2015). Our study shows that circulating T was positively correlated with the chorus's nightly call rates for both calling and non-calling males. This suggests that exposure to conspecific calls could influence circulating androgens in both calling and non-calling males of *L. grylio*. In other species, exposure to conspecific choruses must be paired with gonadotropin
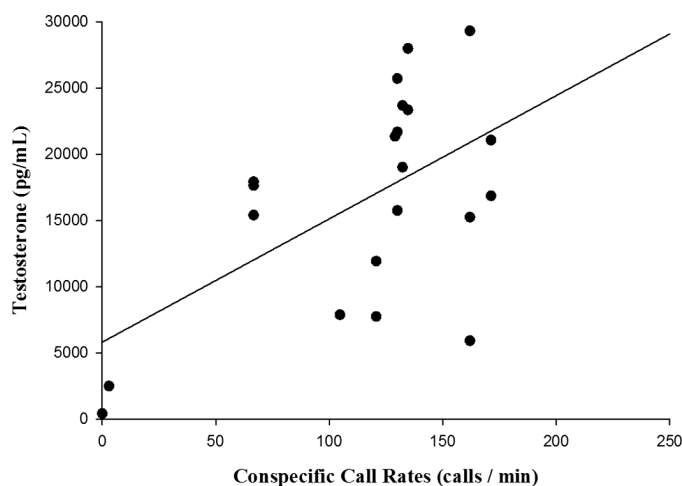

Fig. 5. A linear regression of ambient call rates averaged over one minute and circulating T for individual males. Individual circulating T was positively correlated with the number of conspecific calls recorded during each respective evening of sampling ($R^2 = 0.285$; $\hat{Y}_i = 3895.38 + 31.08X_i$; $P < 0.01$).
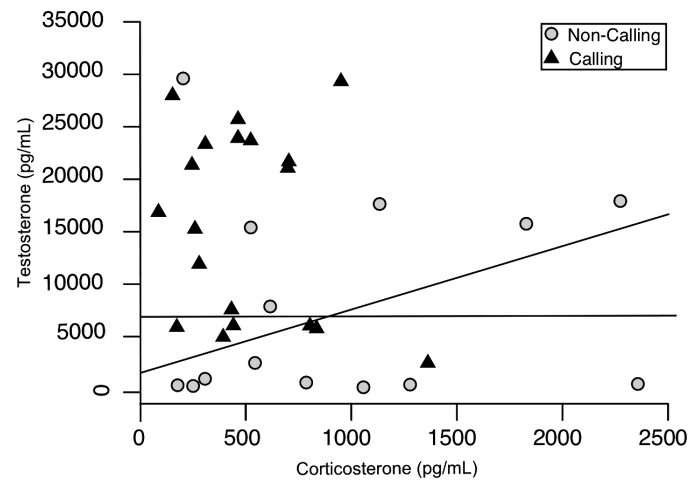

Fig. 6. A scatterplot of circulating CORT and T for calling ($R^2 = 0.04$; $P = 0.38$; $\hat{Y}_i = 18,808.92 - 5.89X_i$) and non-calling males ($R^2 = 0.09$; $P = 0.77$; $\hat{Y}_i = 6,826.35 + 1.09X_i$). These data indicate that CORT does not correlate with circulating T.

treatment to elicit a neural response in auditory processing regions of the brain (Lynch and Wilczynski, 2008; Arch and Narins, 2009). This suggests that T plays a role in altering the sensitivity of the nervous system to auditory reception of chorus vocalizations. Our results suggest that there is a positive relationship between ambient chorus activity and circulating androgens in *L. grylio*.

Corticosterone was higher in non-calling males, but it did not exhibit a seasonal trend. Our data support the EHV model's prediction that elevated CORT concentration could act to suppress calling behavior (Emerson, 2001). Elevated CORT in non-calling males has been found in several anuran species, and treatment with CORT elicited a non-calling state (Burmeister et al., 2001; Leary et al., 2006; Leary and Harris, 2013). Our results indicate that non-calling *L. grylio* have high circulating CORT, low body condition, and that CORT
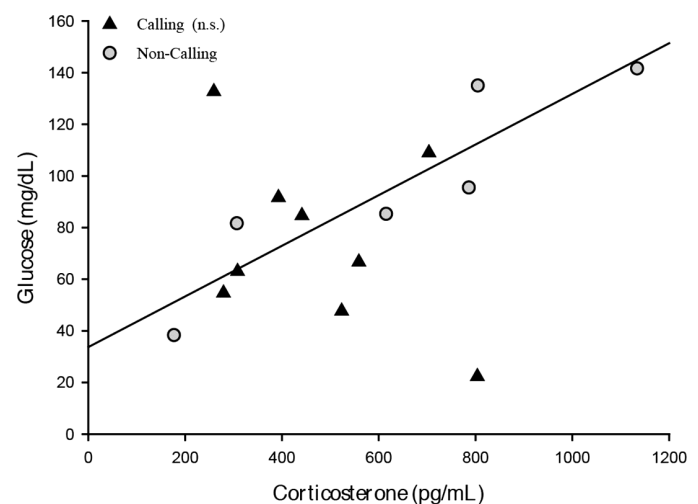

Fig. 7. Linear regression of circulating CORT and glucose concentrations in calling and non-calling males shows the amount of glucose in circulation positively correlates with circulating CORT within non-calling males ($R^2 = 0.665$; $\hat{Y}_i = 31.447 + 0.098X_i$; $P < 0.001$). The regression line is only indicative of a relationship between CORT and glucose concentrations for non-calling males, as there was no significant relationship between CORT and glucose in calling males ($R^2 = 0.149$; $P = 0.305$).

is positively correlated with circulating glucose, which is consistent with the role of CORT in mobilization of energy reserves during a recovery state (Sapolsky et al., 2000). Corticosterone, by action of gluconeogenesis, increases circulating glucose in times of energy expenditure (Wingfield et al., 1990; Emerson, 2001; Romero, 2002). The same CORT–glucose trend would be expected in calling males, but our data did not detect it.

The energetic requirements of calling behavior is hypothesized to cause a spike in CORT, which in turn suppresses said behavior and allows the organism to maintain homeostasis (Emerson, 2001; Leary and Harris, 2013; Leary and Knapp, 2014). However, the results of the current study suggest that CORT and T concentrations fluctuate independently of one another. Independent fluctuation of these hormones is not a novel finding (Leary et al., 2006). One explanation for this phenomenon involves the role of arginine vasotocin (AVT). AVT plays a critical role in the production of vocalizations, and CORT is known to directly inhibit the actions of AVT (Wilczynski and Chu, 2001; Kime et al., 2010; Davis et al., 2015). Therefore, a direct inhibition of AVT by CORT could cause a behavioral shift from calling to non-calling in *L. grylio*. A study manipulating all three hormones (CORT, T, and AVT) would be beneficial in testing this hypothesis.

It is also possible that the effect of CORT on T could be a threshold effect. Corticosterone and T have been hypothesized to increase concomitantly while an individual is calling, until the concentration of CORT reached the threshold where it became suppressive of both T and calling behavior (Leary and Knapp, 2014). At this point, CORT concentration is thought to drop due to the release from the energetic requirements of calling (Emerson, 2001; Leary and Knapp, 2014). Essentially, CORT and T could be both positively (near the onset of calling) and negatively correlated (after the cessation of calling) with one another. This study did not focus on long-term behavioral observations. Therefore, any conclusions that could be made on this phenomenon would be masked by the lack of data indicating individual time spent calling. A study that focuses on circulating hormones as a function of time spent calling would be necessary to test this hypothesis. *Lithobates grylio* would be a difficult species to study for addressing this question due to the fully aquatic and shy nature of this frog.

In summary, calling and non-calling male *L. grylio* exhibited distinct physiological profiles. Glucocorticoids may have played a role in mediating reproductive behavior as suggested by the EHV model, but other hormones should also be considered when researching calling behavior. Testosterone was associated with calling activity, had a seasonal effect, and was correlated with conspecific call rates. Therefore, we conclude that calling behavior in *L. grylio* is associated with both exogenous factors and physiological mechanisms.

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