General and Comparative Endocrinology 213 (2015) 32–39

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

A test of the Energetics-Hormone Vocalization model in the green treefrog

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ARTICLE INFO

Article history:
Received 12 October 2014
Revised 26 December 2014
Accepted 29 December 2014
Available online 6 January 2015

Keywords:
Acoustic signal
Anuran
Courtship signal
Glucocorticoid
Sex steroid

ABSTRACT

Male courtship displays may be regulated by, and affect the production of, circulating hormones. The Energetics-Hormone Vocalization (EHV) model, for example, posits that interactions among chorusing male anuran amphibians stimulate androgen production that then mediates an increase in vocal effort. Increased vocal effort is expected to deplete energy reserves and increase glucocorticoid levels that, in turn, negatively affect androgen levels and vocalization. Androgen levels, glucocorticoid levels, and vocal effort are thus expected to increase across and within nights of chorus activity and should be positively correlated in calling males; energy reserves should decline temporally and be inversely related to glucocorticoid levels. We tested predictions of the EHV model in the green treefrog, *Hyla cinerea*. Consistent with the model, both testosterone and dihydrotestosterone levels increased across the breeding season in calling males. However, testosterone levels decreased and dihydrotestosterone levels did not change within nights of chorus activity, suggesting that chorusing behavior did not drive the seasonal elevation in androgens. Corticosterone (CORT) level remained relatively stable across the breeding season and decreased within nights of chorus activity, contrary to model predictions. Body condition, the proxy for energetic state, was inversely correlated with CORT level but discrepancies between model predictions and temporal patterns of CORT production arose because there was no evidence of a temporal decrease in body condition or increase in vocal effort. Moreover, androgen and CORT levels were not positively correlated with vocal effort. Additional ecological and physiological measures may be needed to support predictions of the EHV model.

1. Introduction

Males of many vertebrate species show pronounced elevations in circulating androgen and glucocorticoid levels during the breeding season (Adkins-Regan, 2005; Goymann et al., 2007; Moore and Jessop, 2003; Romero, 2002). Such seasonal changes in circulating hormone levels may emerge, in part, as a result of competition among males to acquire mates (Adkins-Regan, 2005; Devries et al., 2003; Creel et al., 2013). Agonistic interactions among males, for example, often elevate circulating androgens above breeding baseline levels (e.g., concepts of the "Challenge Hypothesis", Wingfield et al., 1990) while the energetic demands associated with androgen-mediated effects on reproductive behavior potentially elevate circulating glucocorticoid levels (e.g., concepts of the "Energy Mobilization Hypothesis", Romero, 2002). Temporal changes in circulating hormone levels can, in turn, have dramatic effects on male courtship behavior. Moderate elevations in circulating glucocorticoid levels may, for example, promote the expression of energetically costly androgen-mediated courtship signals by mobilizing energy reserves (Buchanan, 2000; Hau et al., 2010; McEwen and Wingfield, 2003; Moore and Jessop, 2003; Ouyang et al., 2013; Romero, 2002) but high levels often inhibit the expression of such signals via negative effects on androgen production (Adkins-Regan, 2005; Greenberg and Wingfield, 1987; Sapolsky, 1992; Sapolsky et al., 2000).

These principles were integrated in the Energetics-Hormone Vocalization (EHV) model to predict temporal patterns of steroid hormone production and vocal behavior in anuran amphibians (Emerson, 2001). The EHV model posits that interactions among chorusing conspecific males (and/or self vocal stimulation, see Cheng, 1992) result in an increase in circulating androgen levels that mediate an increase in vocal effort (i.e., the energy invested in calling behavior). An increase in vocal effort should, in turn, increase the rate of energy depletion (see Wells, 2001 for review on energetic costs of vocalization in anurans) that stimulates the

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http://dx.doi.org/10.1016/j.ygcen.2014.12.017
0016-6480/

production of corticosterone (CORT, the primary glucocorticoid in amphibians). CORT was expected to eventually reach threshold levels (i.e., when energy reserves are depleted) that negatively affect circulating androgen levels and suppress vocal behavior. The EHV model thus predicts that: (1) circulating androgen levels, glucocorticoid levels, and vocal effort will increase across and within nights of chorus activity and should be positively correlated in calling males, (2) energy reserves will decrease across and within nights of chorus activity and should be inversely correlated with CORT level in calling males, and (3) at some point, energy reserves will be depleted and circulating CORT will reach peak levels that suppress androgen production and calling behavior.

The EHV model provides a powerful conceptual framework in which to examine the hormonal basis for variation in anuran vocalizations (Leary, 2009; Wilczynski et al., 2005), and vertebrate courtship behavior in general (Moore and Jessop, 2003). For example, variation in call properties related to vocal effort in anurans (i.e., call duration and/or call rate) can dramatically affect male attractiveness (Gerhardt and Huber, 2002; Ryan, 1985, 1988, 1991) and, hence, an understanding of the hormonal basis for such variation can provide insight into the potential endocrine targets of selection (Adkins-Regan, 2005; Folstad and Karter, 1992; Ketterson et al., 2001; Leary et al., 2006a; Leary and Knapp, 2014). We tested predictions of the EHV model in calling male green treefrogs, *Hyla cinerea,* by examining temporal patterns and the interrelationships among circulating testosterone (T), dihydrotestosterone (DHT), and CORT levels, body condition (the proxy for energy reserves), and vocal effort.

To date, concepts of the EHV model have been most extensively examined in Woodhouse's toad, *Anaxyrus [Bufo] woodhousii*. In this species, androgen levels, vocal effort and body condition remained relatively unchanged and CORT levels decreased across and within nights of chorus activity, contrary to predictions of the EHV model (Leary et al., 2008a). Moreover, androgen level was not positively related to vocal effort. Inconsistencies with model predictions were hypothesized to be related to the explosive breeding pattern that this species exhibits (i.e., males typically call for an average of only 9 nights during the spring and summer months; Leary et al., 2008a,b). For example, in explosive breeders that live in unpredictable environments and mate opportunistically, environmental cues may rapidly stimulate the hypothalamic–pituitary–gonadal axis (Crews and Moore, 1986), thereby creating a ceiling effect where auditory stimulation (Burmeister and Wilczynski, 2000) and/or the production of calls (Cheng, 1992; Cheng and Zuo, 1994) do not cause an additional increase in circulating androgen levels or vocal effort (Leary et al., 2008a). Explosive-breeding species are also less likely to show an inverse relationship between circulating glucocorticoid and androgen levels (i.e., concepts of the Emergency Life-History Stage theory, Wingfield et al., 1995, 1998) that could give rise to inconsistencies with predictions of the EHV model (Leary et al., 2004, 2006a,b, 2008a).

Our recent work suggests that concepts of the EHV model may be more applicable to prolonged-breeding anurans. For example, in *H. cinerea* (a prolonged-breeding species that forms choruses over a period of several months during the spring and summer, see Halliday and Tejedo, 1995), body condition was inversely related to CORT level and non-calling males were in poorer condition and had significantly higher levels of CORT and lower levels of androgens (DHT and T) than calling males (Leary and Harris, 2013). T (but not DHT) level was inversely correlated with CORT level, suggesting that a reciprocal relationship between the two hormones mediates transitions from calling to non-calling behavior, as predicted by the EHV model (Leary and Harris, 2013). Our previous work on *H. cinerea* focused on hormonal differences between male behavioral phenotypes (i.e., calling males and males practicing alternative non-calling "satellite" mating tactics, Leary and Harris, 2013). Here, we examine whether temporal patterns and interrelationships among androgens, CORT, body condition, and vocal effort are consistent with predictions of the EHV model in this prolonged-breeding species. Specifically, social interactions among vocalizing male *H. cinerea* were expected to stimulate temporal elevations in circulating androgen levels (see Burmeister and Wilczynski, 2000) that mediate an increase in vocal effort, deplete energy reserves, and stimulate CORT production. Circulating androgen levels, CORT levels and vocal effort were therefore expected to increase across the breeding season and within nights of chorus activity and be positively correlated in calling males. In contrast, energy reserves were expected to be relatively high at the onset of chorus activity, decrease across and within nights of chorus activity, and be inversely related to CORT level in calling males. Results for this prolonged-breeding species were compared to those previously reported for explosive-breeding *A. woodhousii*.

2. Materials and methods

2.1. Acquisition of data

Choruses of green treefrogs (*H. cinerea*) were studied during the 2011 breeding season at the University of Mississippi Field Station located in Lafayette County, MS. This facility consists of approximately 740 acres of lowland forest with over 200 ponds where *H. cinerea* are abundant. Analysis of hormone levels and body condition was based on data collected from 108 individuals sampled from 12 May 2011 through 28 June 2011; vocalizations were acquired from 73 of those individuals. These dates encompassed the period of chorus activity in 2011. Calling occurred prior and subsequent to these dates but was limited to very few individuals that called sporadically and did not form choruses. Data were collected over the specified time period on all nights of chorus activity and included 12 May, 21–24 May, 28 May–9 June, 11–14, 17, 19–20, 22, 24, 26, and 28 June. "Missing" data represent nights when chorus activity was absent (or nearly so) because of cool or windy weather conditions or severe storms.

Data were collected from chorusing frogs in three ponds located less than 0.5 km from each other; we rotated among these ponds across nights of chorus activity to minimize disturbance to the choruses and to obtain adequate sample sizes (e.g., repeated measures were not acquired from individuals because of the potential effects that repeated sampling has on calling behavior and/or hormone levels). Temporal patterns of chorus activity were nearly identical across the three ponds.

Focal observations were made on calling males using low-powered LED headlamps and, for most individuals, a series of approximately 20 consecutive vocalizations were recorded using a Marantz PMD 222 cassette recorder equipped with a Sennheiser directional condenser microphone (Model ME-66). Individuals were subsequently captured by hand and blood samples (~75 μl) were obtained in less than 2 min via cardiac puncture using a sterile 27 gauge heparinized hypodermic needle and syringe. Individuals were then measured from the tip of the snout to the end of the ischium (snout-ischial length, SIL), weighed, marked on the venter with a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL) and released. Tattoos allowed for future identification and prevented sampling the same individuals more than once (unique tattoo numbers are clearly visible throughout the duration of a single breeding period). Acquisition of blood, body mass/size measures, and application of tattoos was typically completed in 3 min or less and did not appear to have any immediate or long term effects on reproductive behavior; calling individuals typically resumed calling behavior within minutes of release and were often found calling on subsequent nights of chorus activity. Blood samples were stored on ice and subsequently centrifuged at

2200 rpm upon return to the lab (~6 h). Plasma was stored at −20 °C and T, DHT and CORT levels were quantified using radioimmunoassay (described below).

2.2. Column chromatography and radioimmunoassay procedures

Hormone separation and quantification of hormone concentrations followed the protocol described by Knapp and Moore (1995), Knapp et al. (1999), and Leary and Harris (2013). Briefly, plasma samples were incubated overnight with tritiated hormone (PerkinElmer, Inc. Hebron, Kentucky) for determination of recoveries for each sample. Steroids were then extracted from plasma using diethyl ether, dried under nitrogen gas at 40 °C, and resuspended in 10% ethyl acetate in iso-octane. Samples were then loaded onto diatomaceous earth columns containing a 3:1 diatomaceous earth:distilled water "glycol trap" and a 1:1 propanediol:ethylene glycol mixture. Mixtures of 10%, 20%, and 52% ethyl acetate in iso-octane were then used to collect DHT, T and CORT, respectively. Fractions were dried under nitrogen and resuspended in phosphate buffered saline containing 0.3% gelatin for radioimmunoassay. Testosterone antibody was obtained from Fitzgerald Industries International, Inc (Acton, Massachusetts) and used for both T and DHT assays. CORT antibody was purchased from MP Biomedicals, LLC (Solon, Ohio). All samples were assayed in duplicate.

Plasma samples were analyzed for T, DHT, and CORT levels in 4 assays. Assay sensitivities were approximately 0.12 ng/ml for T, 0.13 ng/ml for DHT, and 0.45 ng/ml for CORT. Mean intra-assay coefficients of variation for T, DHT, and CORT were 9%, 14%, and 10%, respectively, based on 3–4 standards run with each assay. Interassay coefficients of variation for T, DHT, and CORT were 18%, 19%, and 19%, respectively.

2.3. Body condition estimates

Body condition estimates were used as a proxy for energy reserves. Body condition was calculated by obtaining the residual values from a linear regression of the cubed root body mass on SIL and dividing those values by the SIL, following the approach described by Baker (1992) and Howard and Young (1998). Such indices are considered valid measures of body condition (Schulte-Hostedde et al., 2005) and have previously been shown to accurately reflect behavior and steroid hormone levels in anurans (Leary et al., 2008a,b; Leary and Harris, 2013).

2.4. Vocal analysis

Calls were analyzed from waveforms and spectrograms using Raven Bioacoustics software (Cornell Bioacoustics Laboratory, Ithaca, NY). We estimated vocal effort based on the average values measured from 7 to 16 consecutive calls for each recorded individual using the following equation:

$$\text{Vocal effort} = \text{call duration}/(\text{call duration} + \text{intercall duration}) \quad (1)$$

Body temperature can alter various vocal parameters in anurans (Brown and Littlejohn, 1972; Gerhardt and Huber, 2002). Temperatures of calling males were measured with a laser-point thermometer (Cen-Tech, Inc) and were used to assess whether temperature was related to vocal effort so that vocal parameters could be corrected accordingly (see Leary, 2001). Vocal effort was not corrected to a common temperature because body temperature was not significantly correlated with vocal effort across the range of temperatures (= 4 °C) measured in chorusing males ($F_{1,109} = 2.18$, $p = 0.14$, $r^2 = 0.02$; analysis based on data from the 73 individuals for which we also had hormone data and an additional 37 individuals for which we had temperature and vocal data only).

2.5. Statistics

2.5.1. Assessing temporal changes in the dependent variables

We measured five dependent variables from calling male *H. cinerea*: (1) plasma T level, (2) plasma DHT level, (3) plasma CORT level, (4) body condition and (5) vocal effort. The two independent variables were: (1) day into the season and (2) time of night, which are both covariates. We assumed that each observation (T level, DHT level, CORT level, body condition, vocal effort, day into season, time of night) was statistically independent and that the dependent variables measured from each frog were correlated. The goal was to compare the correlation coefficients of T level, DHT level, CORT level, body condition and vocal effort, holding fixed the two independent variables (day into season and time of night). But since the independent variables were varying as the individual frogs were collected, we used the following linear model (which allowed us to hold the covariate values fixed) as the starting point to assessing temporal changes in the dependent variables:

$$\begin{aligned}\text{Yikrt} = {} & \text{b0}i + (\text{b1}i)(\text{day into season\_k}) + (\text{b2}i) \\ & \times (\text{time of night\_}r) + (\text{b3})(i = 1) \\ & \times (\text{time of night\_}r^2) + (\text{b4})(i = 4) \\ & \times (\text{time of night\_}r^2) + \text{Eikrt}\end{aligned} \quad (2)$$

where Yikrt denotes any of the dependent variables $i = 1, \ldots, 5$ (where 1 = T level, 2 = DHT level, 3 = CORT level, 4 = body condition and 5 = vocal effort), k indexes day into season (e.g., across days of chorus activity), r indexes time (e.g., across hours of the night) and t denotes a single frog; Eikrt is thus an error term (for a single frog, t). For T level and body condition, extra terms (time of night_r^2) were added to the model because the data plots indicated the need for quadratic terms.

Before any temporal correlation analysis could be performed on the model described in Eq. (2), the five dependent variables were first transformed until their joint distribution was as close to normal as possible. We did this by performing ordinary least squares regressions against day into season and time of night using the model shown in Eq. (2), but restricting the model to only one dependent variable at a time. We then analyzed the residual values and transformed the data when necessary (see Neter et al., 1996). As a result, T and CORT levels were log-transformed and DHT level was square-root transformed. We then fit the model shown in Eq. (2) with Proc Mixed in SAS (SAS Institute, Cary, North Carolina, USA) to analyze seasonal and nightly changes in the dependent variables.

Note that the five dependent variables were measured on each frog simultaneously and were thus correlated. Hence, the error matrix (Eikrt: $i = 1, 2, 3, 4, 5$) in Eq. (2) was also correlated, and was thus applied as unstructured (Proc Mixed in SAS, SAS Institute, Cary, North Carolina, USA). Specifically, since the two independent variables (day into season and time of night) are viewed as non-random, it follows that variance (Yikrt) = variance (Eikrt) for $i = 1, \ldots, 5$ from the model described in Eq. (2). Hence, for one frog, t, the covariance matrix was a 5 × 5 matrix (for the dependent variables 1 = T level, 2 = DHT level, 3 = CORT level, 4 = body condition and 5 = vocal effort). That matrix was as follows:

$$\text{Var}(\text{Yikrt}: i = 1,2,3,4,5) = \begin{pmatrix} \text{sig}^{**}2\_1 & \text{cov}(Y1,Y2) & \text{cov}(Y1,Y3) & \text{cov}(Y1,Y4) & \text{cov}(Y1,Y5) \\ & \text{sig}^{**}2\_2 & \text{cov}(Y2,Y3) & \text{cov}(Y2,Y4) & \text{cov}(Y2,Y5) \\ & & \text{sig}^{**}2\_3 & \text{cov}(Y3,Y4) & \text{cov}(Y3,Y5) \\ & & & \text{sig}^{**}2\_4 & \text{cov}(Y4,Y5) \\ & & & & \text{sig}^{**}2\_5 \end{pmatrix} \quad (3)$$

where we assume that all the frogs in the data set have the same covariance matrix. The fact that the covariance matrix is necessarily symmetric (a fact about the variance (Yikrt) matrix) leads to a total of 15 parameters: 5 variances for $i = 1, \ldots, 5$, and 10 covariances of the above-diagonal elements (recall the covariances are non-zero due to correlation). We assume that all elements of (Eikrt: $i = 1, 2, 3, 4, 5$) are random variables, are normal with mean = 0, and with variance–covariance matrix given by Eq. (3). On the diagonal elements $(i, j) = (1, 1), (2, 2), \ldots, (5, 5)$ of variance (Yikrt), we thus have variance (Yikrt) = [sig^2_i], if i = variables 1, …, 5. The non-diagonal elements of variance (Yikrt) are covariance (Yi, Yj), which are possibly different and distinct for each $i > j$. The term "unstructured" thus refers to one (of many) covariate-variance setups which are possible with Proc Mixed. The parameter estimates and standard errors of b0i, b1i, b2i, b3 and b4 generated from the model described in Eq. (2) and Proc Mixed are provided in Table 1.

Each point in Fig. 1 (see Supplementary Data) corresponds to one frog, and "days into season" and/or "time of night" differ for each frog in the data set. We thus used the following covariate values: day into season = 12, 26, 40 days and time of night = 2200 h to examine how the dependent variables changed temporally. The day into season values approximated the 10th, 50th and 90th quantiles of the distribution (Fig. 1 in Supplementary Data). The time of night value (2200 h) approximated the median time that the frogs were observed on a nightly basis (Fig. 1 in Supplementary Data). We used Proc Mixed's "Contrast" method (SAS Institute, Cary, North Carolina, USA) to generate estimates for the dependent variables (Table 2). For instance, for the model described in Eq. (2) and for one dependent variable at a time (e.g., let $i = 1$ = T level), we took covariate one (day into season = 12) and covariate two (time of night = 2200) and formed the hypotheses:

$$H_0 : b01 + (b11)(12) + (b21)(2200) = 0$$

$$H_1 : b01 + (b11)(12) + (b21)(2200) \neq 0$$

Hence, for the current example, if H_0 is true, it means that T level (= variable 1) is not different from 0 due to chance under the model described in Eq. (2) (i.e., the measured parameter did not change temporally). Rejection of H_0 indicates that T level is not equal to 0 under the same model (i.e., the measured parameter changed temporally). The Proc Mixed procedure then computes a p-value (shown on Table 2), which is the result for that combination of covariates (note that H_0 and H_1 will change across the rest of the 14 combinations shown in Table 2). For this same example using T level, results provided in Table 2 indicated that the H_0 was rejected (e.g., $p < 0.0001$), and the estimates provided in Table 1 were b11 = 0.15 ($p = 0.0002$) and b21 = −2.73 ($p = 0.03$). So for dependent variable 1 (T level), and the two covariates (days = 12 and time = 2200), the model described in Eq. (2) indicates that T level increased across days of the breeding season (held fixed at days = 12) by 0.15, and decreased within nights of chorus activity (held fixed at night = 2200) by −2.73. This procedure was carried out for all dependent variables and the designated levels of the covariate values.

Table 1
Results generated from Proc Mixed procedures using the appropriately transformed variables measured in calling male *Hyla cinerea* (log T = log transformed testosterone level (ng/ml), √DHT = square-root transformed dihydrotestosterone level (ng/ml), log CORT = log transformed corticosterone level (ng/ml), BC = body condition, and VE = vocal effort). "Estimate" values are the parameter estimates for each variable that were used in the model described in Eq. (2) to ascertain how the measured variables changed temporally when the covariate values for day into season and time of night were held fixed (see Table 2). Values in bold represent significant seasonal (day of season) or nightly (time of night) relationships.

Variable effect	Estimate	Standard error	t-Value	$pr > \|t\|$
log T	33.14	14.80	2.24	**0.02**
√DHT	0.419	2.12	0.20	0.84
log CORT	18.40	2.93	6.28	**<0.0001**
BC	−0.128	0.068	−1.89	0.06
VE	0.419	0.138	3.03	**0.003**
Day of season * log T	0.15	3.83Ee−3	3.93	**0.0002**
Day of season * √DHT	0.017	6.8e−3	2.43	**0.01**
Day of season * log CORT	−0.005	1.4e−2	−0.37	0.71
Day of season * BC	−7.41e−6	1.4e−5	−0.53	0.60
Day of season * VE	6.0e−5	4.3e−4	0.14	0.88
Time of night * log T	−2.73	1.31	2.08	**0.03**
Time of night * √DHT	0.147	0.095	1.52	0.13
Time of night * log CORT	−0.802	0.131	−6.12	**<0.0001**
Time of night * BC	0.011	6.1e−3	1.82	0.07
Time of night * VE	−0.008	6.1e−3	−1.32	0.18

2.5.2. Assessing contrasts among the dependent variables

Relationships among the 5 dependent variables were examined using contrasts on the values obtained from the model described in Eq. (2). The contrasts were of the form of hypotheses described above.

2.5.3. Controlling for Type I error rates

A Bonferroni correction was applied to control for Type I error rates. For analysis involving temporal changes in the dependent variables (described above), the adjusted alpha value (= 0.003) was calculated by dividing alpha (= 0.05) by the number of contrasts (= 15) shown in Table 2.

For the residuals of fitting the five dependent variables with Proc Mixed by Eq. (2), we obtained the 10 estimated Pearson correlation coefficients (shown in Table 3) to control. Hence, alpha (= 0.05) was divided by 10 to calculate the adjusted alpha value (= 0.005).

We note that strict adherence to Bonferroni corrections can increase the probably of Type II error rates (see Nakagawa, 2004). We were thus cautious not to entirely dismiss findings that were significant prior to, but not subsequent to, Bonferroni correction.

2.6. Collecting permits and IACUC approval

Scientific collecting permits were provided by the Mississippi Department of Wildlife, Fisheries and Parks. All procedures were approved by the University of Mississippi Animal Care and Use Committee.

3. Results

3.1. Seasonal and nightly changes in the measured variables

3.1.1. Androgens

Estimates for both T level and DHT level were rejected by H_0 for days 12, 36 and 40, regardless of whether a Bonferroni correction was applied or not ($p < 0.0001$ in all cases, Table 2). In other words, results indicated that T and DHT levels changed temporally (e.g., across days of season and/or across hours of the night). For T level, estimate values increased across days 12, 26 and 40 (= 2.12, 2.32 and 2.54, respectively; Table 2). Results were attributable to a significant and positive seasonal increase in circulating T levels (covariate estimate = 0.15, $p = 0.0002$, Table 1) that outweighed a significant (but weaker) nightly decline in T levels (covariate estimate = −2.73, $p = 0.03$, Table 1). For DHT level, estimates also increased across days 12, 36 and 40 (= 3.80, 4.03 and 4.27, respectively; Table 2) and were primarily attributable to a seasonal increase in DHT levels (covariate estimate = 0.017, $p = 0.01$, Table 1); there was no evidence that DHT varied within nights of chorus activity (covariate estimate = 0.147, $p = 0.13$, Table 1).

Table 2
Model results for the five dependent variables measured in male *H. cinerea*: (1) log testosterone (T) (ng/ml), (2) square root dihydrotestosterone (DHT) (ng/ml), (3) log corticosterone (CORT) (ng/ml), (4) body condition, and (5) vocal effort. "Estimate" values were calculated by setting the covariate values to day 12, 26, and 40 (for day of season) and time to 2200 h (for time of night) in Eq. (2) (see text). Significant *p*-values (alpha = 0.05) are in bold and those that remain significant after a Bonferroni correction was applied (alpha = 0.003) are indicated with an asterisk. When combined with Table 1, the results allow for determination of how the dependent variables changed across the breeding season and/or within nights of chorus activity (see text).

Day	Time	Variable	Estimate	Standard error	*p*-Values
12	22:00 h	log T	2.12	0.07	**<0.0001***
		√DHT	3.80	0.14	**<0.0001***
		log CORT	0.70	0.24	**0.004**
		Body condition	2.5e−4	2.5e−4	0.32
		Vocal effort	0.24	0.007	**<0.0001***
26		log T	2.32	0.05	**<0.0001***
		√DHT	4.03	0.08	**<0.0001***
		log CORT	0.63	0.14	**<0.0001***
		Body condition	1.5e−4	1.7e−4	0.36
		Vocal effort	0.24	0.004	**<0.0001***
40		log T	2.54	0.07	**<0.0001***
		√DHT	4.27	0.14	**<0.0001***
		log CORT	0.55	0.23	**0.02**
		Body condition	4.7e−5	2.54e−4	0.85
		Vocal effort	0.24	0.008	**<0.0001***

Table 3
Pearson correlation results among all measured variables in calling male *Hyla cinerea*. log T = log transformed testosterone level (ng/ml), √DHT = square root-transformed dihydrotestosterone level (ng/ml), log CORT = log transformed corticosterone level (ng/ml). Correlation coefficients are presented above *p*-values (in italics). Significant correlations (alpha = 0.05) are in bold and those that remain significant after a Bonferroni correction was applied (alpha = 0.005) are indicated with an asterisk. Sample size = 72 for correlations involving vocal effort and 107 for all other correlations.

	log T	√DHT	log CORT	Body condition	Vocal effort
log T	1.0	0.710	−0.134	0.200	0.156
		<.0001*	*0.17*	***0.03***	*0.19*
√DHT		1.0	0.034	0.073	0.152
			0.72	*0.46*	*0.20*
log CORT			1.0	−0.273	0.071
				0.004*	*0.55*
Body condition				1.0	0.039
					0.74
Vocal effort					1.0

3.1.2. Corticosterone

Estimates for CORT level were rejected by H_0 for days 12 ($p = 0.004$), 36 ($p < 0.0001$) and 40 ($p = 0.02$) prior to Bonferroni correction; only day 36 differed from zero subsequent to Bonferroni correction (Table 2). Results suggested that there was some evidence of seasonal decline in CORT level (i.e., estimate values across days 12, 36 and 40 were 0.70, 0.63 and 0.55 respectively, see Table 2) but that this seasonal decline was weak (covariate estimate = −0.005, $p = 0.71$, Table 1). In contrast, there was strong evidence that CORT level declined within nights of chorus activity (covariate estimate = −0.802, $p < 0.0001$, Table 1). Apparently, CORT does not return to the same (relatively high) level at the onset of subsequent nights of chorus activity (i.e., after a nightly decline in CORT level), thereby contributing to a weak seasonal decline.

3.1.3. Body condition

There was no evidence that body condition varied temporally ($p \geqslant 0.32$, Table 2; $p \geqslant 0.07$, Table 1).

3.1.4. Vocal effort

Vocal effort was rejected by H_0 for days 12, 36 and 40 regardless of whether a Bonferroni correction was applied or not ($p < 0.0001$ in all cases, see Table 2) suggesting that vocal effort varied temporally. However, there was no evidence that vocal effort changed across days 12, 36 and 40 (estimate values = 0.24 for all days, Table 2) and no evidence of a significant seasonal or nightly effect ($p \geqslant 0.18$, Table 1). Our interpretation of these results is that the estimates for vocal effort were consistently higher than zero but there was no evidence of a temporal change in vocal effort.

3.2. Correlation analysis of the residuals from the model in Eq. (2)

Interrelationships among the 5 dependent variables are summarized in Table 3. The only variables that were significantly correlated after a Bonferroni correction were T and DHT levels (which were positively correlated, unadjusted $p < 0.0001$), and body condition and CORT level (which were negatively correlated, unadjusted $p = 0.004$). Circulating T level and body condition were positively but weakly correlated (i.e., there was a significant correlation prior to, but not after, a Bonferroni correction was applied, unadjusted $p = 0.03$).

4. Discussion

Analysis of temporal patterns and interrelationships among T levels, DHT levels, CORT levels, body condition, and vocal effort in *H. cinerea* provided two lines of evidence supporting predictions of the EHV model (Emerson, 2001). First, both T and DHT levels increased across the breeding season. Second, body condition was inversely correlated with circulating levels of CORT, suggesting that the depletion of energy reserves stimulates CORT

production. However, none of the other predictions of the EHV model were supported and, in some cases, results were opposite of model predictions. For example, the EHV model predicts that CORT levels will increase across and within nights of chorus activity but there was no evidence that CORT level increased across the breeding season and CORT levels actually decreased within nights of chorus activity in calling male *H. cinerea*.

It was surprising that many key predictions of the EHV model were not supported. For instance, vocal production in anurans is undoubtedly energetically demanding (reviewed by Wells, 2001) and is expected to deplete males of energy reserves. In *H. cinerea*, however, there was no evidence that body condition of calling males declined temporally (i.e., seasonally or nightly). Apparently, calling males acquire enough food across and within nights of chorusing behavior to maintain relatively constant body condition. Hence, circulating CORT levels did not increase temporally (i.e., seasonally or nightly). Similar results were also found for explosive-breeding *A. woodhousii* (Leary et al., 2008a). Interestingly, in both *H. cinerea* and *A. woodhousii*, CORT levels actually decline in calling males within nights of chorus activity. In *H. cinerea*, such a pattern may be related to temporal variation in aggressive interactions. For example, the frequency of aggressive interactions in male *H. cinerea* is often highest at the onset of nightly chorus activity when males are establishing territorial boundaries (Oldham and Gerhardt, 1975) and such interactions have been shown to stimulate CORT production (Leary, 2014). Hence, CORT levels may peak at the onset of nightly chorus activity and subside as nightly chorus activity and the frequency of aggressive interactions wane. It is not currently clear why such a pattern also exists in *A. woodhousii*, which is not territorial nor does it produce any distinct aggressive acoustic signals (Leary et al., 2004). However, advertisement calls can also stimulate CORT production in male anurans (see Leary, 2014) and may account for similar patterns of CORT production in both species. Increased chorus density is expected to increase the frequency of agonistic interactions and can stimulate CORT production in *A. woodhousii* (Leary et al., 2008b), but we have not measured temporal variation in chorus density in *H. cinerea* to assess how it potentially contributes to the nightly decline in CORT levels.

One central prediction of the EHV model is that social interactions among chorusing males drive temporal elevations in circulating androgen levels (based on concepts of the Challenge Hypothesis, Wingfield et al., 1990). While androgen levels clearly increased across the breeding season in *H. cinerea* (as predicted by the EHV model), it is not currently clear whether this pattern was attributable to interactions among chorusing males or some other abiotic factor that may modulate androgen production. For example, if seasonal elevations in circulating androgens were the result of interactions among chorusing males, we would expect to find evidence that androgen levels increase during nighttime periods of chorus activity (i.e., when males are interacting). However, there was no support for this hypothesis in *H. cinerea*; circulating T levels actually decreased within nights of chorus activity and DHT levels remained relatively stable over the same time frame. Hence, for a seasonal increase in androgen levels to occur, androgen levels must be increasing during the day when animals are not chorusing. These findings contrast with predictions of the EHV model, which proposes that nightly elevations in androgen levels will be followed by a decrease (i.e., clearance) of androgens during the day; the rate of nighttime production was expected to exceed daytime clearance rates, resulting in a seasonal increase on androgens. While broadcast chorus sounds can stimulate androgen production in *H. cinerea* (see Burmeister and Wilczynski, 2000), such effects may not translate into nightly elevations in circulating androgen levels in natural choruses because close-range aggressive vocal exchanges (which occur frequently in natural choruses) can decrease androgen levels in this species (see Leary, 2014).

In *A. woodhousii*, there was also no evidence that androgen levels increased within nights of chorus activity, nor was there evidence that androgens increase across the breeding season (Leary et al., 2008a). In this explosive-breeding species, we proposed that environmental cues (e.g., rainfall) rapidly stimulate the hypothalamic–pituitary–gonadal axis, thereby creating a ceiling effect where auditory stimulation does not cause an additional increase in circulating androgen levels (Leary et al., 2008a). In *H. cinerea*, abiotic environmental cues also appear to modulate androgen levels, resulting in seasonal elevations in circulating androgen levels. It is not currently clear why androgen levels increase across the breeding season in *H. cinerea* and not *A. woodhousii,* but it is possible that explosive-breeding species are more responsive to such cues and thus attain maximum androgen levels more rapidly.

Our data also did not support several other key predictions of the EHV model. For example, the model predicts that a temporal increase in androgen levels will mediate a concordant temporal increase in vocal effort that increases the rate of energy depletion and the production of CORT. Hence, circulating levels of androgens and CORT should both be positively correlated with vocal effort in calling males. Although androgen level increased across the breeding season in *H. cinerea*, there was no evidence of a seasonal increase in vocal effort. Moreover, neither androgen level nor CORT level were positively correlated with vocal effort. There was also no evidence that vocal effort was positively correlated with circulating androgen levels in *A. woodhousii*, although CORT level was positively correlated with vocal effort in this species (Leary et al., 2008a).

Graded effects of androgens on vocal effort in anurans, as proposed by the EHV model, are controversial (reviewed by Leary, 2009). Androgens more often appear to play a permissive role in male sexual traits wherein androgens mediate the expression of the trait but variation in circulating levels does not reflect variation in the extent or magnitude of the trait (see reviews by Hews and Moore, 1997; Adkins-Regan, 2005; Leary and Knapp, 2014). The results presented here suggest that variation in vocal effort among calling male *H. cinerea* is not related to variation in circulating androgen levels (see also Burmeister and Wilczynski, 2001). In contrast, the lack of a significant relationship between CORT level and vocal effort in *H. cinerea* is likely to be attributable to the multitude of intrinsic and extrinsic factors that potentially modulate circulating CORT levels (Sapolsky, 1992; Sapolsky et al., 2000; Wingfield and Ramenofsky, 1999). For example, predation by snakes (particularly cottonmouths, *Agkistrodon piscivorous*) was commonly observed at our study sites and detection of such predators could dramatically increase CORT production (see Narayan et al., 2013). Moreover, chorus density and aggressive acoustic interactions among males can stimulate CORT production (Leary et al., 2008b; Leary and Harris, 2013; Leary, 2014). Such effects could effectively decouple circulating CORT levels from body condition estimates and/or vocal effort in *H. cinerea*. Variation in steroid binding proteins and/or receptors levels may further complicate these relationships.

A primary concern associated with testing concepts of the EHV model involves the level of analysis that is appropriate for addressing its predictions. For example, analysis of circulating hormone levels and body condition estimates in individual calling males and non-calling males practicing an alternative "satellite" mating tactic in *H. cinerea* are consistent with predictions of the EHV model; satellite males are in poorer condition and have higher CORT levels and lower androgen levels than calling males (Leary and Harris, 2013). However, we do not know whether satellite males represent a class of individuals in poor condition with chronically elevated CORT levels and low androgens or whether these factors

change during transitions between calling and satellite behavior (i.e., longitudinal data are lacking, Leary and Harris, 2013). Such data are obviously important for testing temporal predictions of the EHV model. One possibility is that the measured variables (reported here) do not vary temporally in a manner that is consistent with model predictions because individual males rapidly experience changes in hormone levels (reciprocal interactions between glucocorticoids and androgens) and calling behavior (transitions between calling and non-calling behavior) at different times throughout the breeding period. This potential problem is analogous to temporal hormone–behavior relationships reported in birds. For example, the relationship between T level and aggression in birds was not well understood until the temporal data (initially organized by calendar date) was reanalyzed in the context of the phase of the breeding cycle (see Hegner and Wingfield, 1986; Wingfield et al., 1987). Similar analysis may prove to be difficult in anurans that rapidly alternate between calling and non-calling behavior within and across nights of calling behavior. Similarly, it may be particularly difficult to use a repeated measures design in anurans because of the likelihood that repeated acquisition of blood samples will cause changes in hormone levels and calling behavior.

5. Conclusions

While circulating androgens and glucocorticoids are often elevated during the reproductive period in anurans and other vertebrates (Eikenaar et al., 2012; Goymann et al., 2007; Licht et al., 1983; Moore and Jessop, 2003; Romero, 2002), patterns of hormone production within the breeding season and the interrelationships among the various factors associated with the EHV model are less clear. For example, while some of the variables were interrelated in ways that were predicted by the EHV model (e.g., body condition was inversely related to CORT level), the lack of a strong positive relationship between and among key variables (e.g., androgen level, CORT level, and vocal effort) affected nearly all other predictions of the model. Hence, although concepts of the EHV model are logically interconnected, they are not well supported by current data on explosive-breeding or prolonged breeding anuran species. We clearly need additional studies to assess why such discrepancies arise and to formulate new models regarding the hormonal basis for variation in calling behavior in anurans. Additional measures of binding proteins and receptor levels may be particularly important in understanding the endocrine factors mediating variation in such behavior.

Acknowledgments

We thank A.G. Davis, S. Crocker-Buta and R. Knapp for providing comments on an early version of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.12.017.

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