Mutually reinforcing hormone–behaviour relationships may drive temporal changes in steroid hormone levels and vocal effort in anuran amphibians. Recent models propose that chorus activity stimulates the production of androgens in signallers and receivers, thereby mediating an increase in vocal effort. The energetic demands associated with high vocal effort should, in turn, elevate circulating corticosterone levels to promote the mobilization of energy reserves. High threshold corticosterone levels, however, are expected to negatively affect androgen levels and, hence, vocal effort. Steroid hormone levels and vocal effort are thus expected to be low at the onset of chorus activity, increase over sequential nights of calling until peak levels are reached, and subsequently decline; energy reserves should show inverse temporal trends. To test these predictions, we examined temporal changes in androgen (dihydrotestosterone and testosterone) levels, corticosterone levels, vocal effort and body condition in Woodhouse’s toads, *Bufo woodhousii*. Contrary to model predictions, androgen levels, vocal effort and body condition remained relatively unchanged and circulating corticosterone levels tended to decrease within and across nights of chorus activity. Furthermore, although vocal effort was not correlated with circulating androgen levels, it was positively correlated with circulating corticosterone levels, supporting the prediction that high vocal effort promotes the production of corticosterone. Yet despite this correlation, poor body condition was not associated with high corticosterone levels, suggesting that factors other than energetic state influence corticosterone levels in *B. woodhousii*. We discuss alternative hypotheses regarding relationships among the measured parameters in this explosive-breeding species.

Hormone levels can greatly influence the expression of sexually selected traits. Males’ sex steroid levels, in particular, have been the focus of most research given their potential influence on courtship display rates and attractiveness (reviewed by Adkins-Regan 2005). For example, male dark-eyed juncos, *Junco hyemalis*, treated with testosterone sing more and are preferred by females (Enstrom et al. 1997; Ketterson & Nolan 1999). However, the considerable amount of energy often invested in courtship displays (Andersson 1994) may stimulate the production of glucocorticoids to promote the mobilization of energy reserves (Romero 2002; McEwen & Wingfield 2003). Glucocorticoids can, in turn, suppress activity of the hypothalamic–pituitary–gonadal (HPG) axis (Greenberg & Wingfield 1987; Sapolsky 1992a, b; Wingfield et al. 1998), thereby altering the expression of androgen-mediated courtship displays (reviewed by Adkins-Regan 2005).
Calling behaviour in male anuran amphibians (frogs and toads) is particularly well suited for studying the links among hormone levels, energy expenditure and reproductive behaviour. Vocal production by anurans is androgen dependent (reviewed by Moore et al. 2005; Wilczynski et al. 2005) and energetically demanding (Bucher et al. 1982; Pough et al. 1992; Prestwich 1994; Wells 2001) and males often show pronounced elevations in corticosterone levels during the reproductive period (Licht et al. 1983; Romero 2002; Woolley et al. 2004). Elevated corticosterone levels, in turn, have an inhibitory effect on calling behaviour in anurans, potentially via negative effects on circulating androgen levels (Mendonça et al. 1985; Marler & Ryan 1996; Burmeister et al. 2001).

Emerson (2001) provided a conceptual model that integrates relationships among androgen levels, corticosterone levels, vocal effort and energy reserves to explain transitions between calling and noncalling behaviour in male anurans as well as temporal changes among these factors. For example, the energetics hormone-vocalizations (EHV) model (Emerson 2001) proposes that the transition between calling and noncalling behaviour is mediated by the high energetic requirements associated with vocal production that drives temporal elevations in circulating corticosterone. Levels of circulating corticosterone are predicted to reach a threshold (when energy stores are depleted) that negatively affect circulating androgen levels and, hence, calling behaviour. We previously examined whether such hormonal interactions explained transitions between calling and noncalling behaviour in Woodhouse’s toads, Bufo woodhousii, and Great Plains toads, B. cognatus. We found that calling male toads had significantly higher corticosterone levels than did noncallers, but androgen levels did not differ between the two groups (Leary et al. 2004). In addition, experimental manipulation of corticosterone levels indicated that the transition between calling and noncalling behaviour was mediated by high corticosterone levels, but changes in behaviour occurred independently of changes in androgen levels (Leary et al. 2006a). These results are, in part, consistent with predictions of the EHV model; corticosterone mediates transitions between calling and noncalling behaviour, but does not have the predicted suppressive effect on androgen levels. Here, we examine additional aspects of the EHV model. Specifically, we address predictions regarding temporal changes in, and relationships among, androgen levels, corticosterone levels, vocal effort and body condition (the proxy for energy reserves) in calling male B. woodhousii.

The EHV model predicts that androgen levels, corticosterone levels, vocal effort and energy reserves will vary temporally and be highly correlated in calling male anurans. For example, the model posits that androgen and corticosterone levels should be lowest at the onset of yearly chorus activity and increase from some baseline level in calling males within and across nights of breeding activity. Androgen levels are expected to increase over time in response to reproductive behaviours of other conspecific males in the chorus (based on concepts of the challenge hypothesis, Wingfield et al. 1990) and/or through self-stimulation of the hypothalamic–pituitary–gonadal (HPG) axis (e.g. Cheng 1992). Circulating corticosterone levels are also expected to show concordant temporal changes because of the high energetic requirements associated with androgen-mediated changes in vocal effort. Emerson (2001) predicted that such temporal changes in steroid hormone levels should be detectable over multiple temporal scales, including within a single evening of chorus activity as well as across the breeding season. The model proposes that these patterns occur over the longer temporal scales because steroid hormone levels were not expected to return to baseline levels during the interim of inactivity between nights of calling (during periods of daytime inactivity), resulting in seasonal temporal elevations in steroid hormone levels that peak and subsequently decline over time (Emerson 2001).

The EHV model thus predicts that robust correlations will exist among androgen levels, corticosterone levels, vocal effort and energy reserves in calling males. For instance, the model posits that elevated androgen levels will promote an increase in vocal effort, resulting in the depletion of energy reserves and elevated corticosterone levels. Hence, corticosterone and androgen levels should be positively correlated with vocal effort, but corticosterone levels should be negatively correlated with available energy reserves in calling males (Emerson 2001). Temporal changes in hormone levels should therefore track changes in vocal effort and energy reserves.

Such relationships are clearly important in understanding the physiological factors underlying variability in signal attractiveness (high vocal effort is often preferred by female anurans, reviewed by Gerhardt & Huber 2002). To date, however, no study has concurrently examined these factors in any anuran to determine whether the model accurately predicts temporal changes and relationships among steroid hormone levels, vocal effort and energy reserves. The present study examines relationships among these factors over four consecutive breeding seasons in calling male Woodhouse’s toads.

**METHODS**

**Study Site**

Our study pond was located in central Oklahoma (Cleveland County) on a privately owned horse pasture. Chorus activity was monitored within a 10 km radius of the study site almost every night (often at multiple times each night) throughout the spring and summer months for 4 consecutive years (2001–2004). The area was monitored less frequently when extreme environmental conditions prevailed (i.e. near-freezing temperatures, when the species is inactive) or when potential breeding sites lacked standing water (the species only breeds when ephemeral pools are filled). Toads in this population did not call during the day. One other chorus was found across all years approximately 3.2 km from the study site and never consisted of more than 12 individuals. Marked individuals from our study pond were never found at this nearby site. Given our monitoring protocol, we are confident that patterns of chorus activity across years reflect the complete breeding activity periods for these years.
Patterns of chorus activity varied considerably within years (between the months of April and June) and across years (Table 1). Data were collected throughout each night of chorus activity for the entire breeding season for all years with the exception of 10 April 2001 when severe lightning storms prevented data collection even though a large number of individuals were actively calling. Data were also not collected on 19–23, 26 and 27 April 2003 because on these nights only four or fewer individuals arrived at the breeding site (4, 2, 2, 1, 1, 1 and 3 individuals, respectively). These 7 nights were characterized by high winds and low temperatures and males either did not call or called only sporadically before leaving the site within an hour after arrival.

The patterns of chorus activity also varied within nights. However, males typically arrived at the site after sunset (~2100 hours) and began leaving between 0300 and 0600 hours. Data were collected continuously throughout the evening, beginning within an hour of the onset of chorus activity (i.e. after a notable decrease in incoming migrants was observed) and ending when a large proportion of calling individuals were observed leaving the pond, which often occurred relatively rapidly (within an hour; ampliced, non-calling individuals often remained at the pond until dawn). In some cases, data collection (and/or chorus activity) was interrupted within a single night of activity by dangerous weather conditions. Our study site lies within ‘Tornado Alley’, and severe winds, heavy rains, hail and lightning storms can arrive rapidly. Such storms are often brief, and toad breeding activity may either continue, be interrupted and subsequently resume, or abruptly stop for that evening.

### Data Collection

Behavioural observations of calling males were made using flashlights, low-powered LED headlamps, or under ambient moonlight conditions. Artificial light did not have any overt effects on behaviour (see also Sullivan 1989). Recordings of 6–10 consecutive vocalizations acquired from individual toads were made using Marantz PMD-222 recorders equipped with Sennheiser ME66 condenser microphones. Call amplitude was also measured from a subset of recorded individuals using RadioShack digital sound pressure level (SPL) meters (Model 33-2055). A 30 cm, heavy gauge wire was mounted on the SPL meters to control for changes in amplitude readings associated with variation in the distance between the subject and SPL meter. Calling individuals were approached and the wire mount on the SPL meter was oriented horizontal to the water substrate and placed directly in front of (almost touching) the calling male’s expanded gular sac to obtain SPL readings (Leary et al. 2006b). Individuals were then immediately captured by hand and a blood sample was rapidly obtained by cardiac puncture. Blood was collected in 3 min or less in 2001 and in less than 30 s in subsequent years. Blood samples were kept on ice until they were returned to the laboratory (<8 h). There, samples were centrifuged for 12 min at 3000 revolutions/min and the plasma was stored at −20°C until assayed for steroid hormones.

Calling males were then measured from the tip of the snout to the distal end of the ischium (to the nearest 1 mm) and weighed (to the nearest 0.1 g) on a portable OHAUS digital scale after gently drying the individual with a cloth. All sampled males were then numerically marked on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, U.S.A.). Tattoos allowed identification throughout an entire breeding season, but not between years (the mark was not clearly visible the following breeding season). Data were undoubtedly acquired from many of the same individuals as well as from new cohorts of individuals across years because males participating in chorus activity in this population ranged between 1 and 6 years of age (Leary et al. 2005). All handling took ~2 min (~5 min in 2001) and did not significantly affect circulating androgen or corticosterone levels over a 1 h period (Leary et al. 2006a). Males were then returned to their site of capture where they typically resumed calling within 1 min of release. In some instances, two blood samples were obtained from the same individual at various time intervals (i.e. within an evening, across consecutive evenings, or across the breeding period), in which case the same procedures as described above were followed.

All methods and procedures were approved by the University of Oklahoma Institutional Animal Care and Use Committee (R01-005). In addition, a veterinarian (A.M.G.) was present to monitor the animals on all nights of the study.

### Vocal Analysis

Calls were analysed using Canary 1.1.1 Bioacoustics software (Cornell Bioacoustics Laboratory, Ithaca, NY, U.S.A.). We examined call parameters from spectrograms in bufonids and other anurans (Gerhardt & Huber 2002) or that were required to estimate vocal effort. Call duration (s), pulse rate (the number of pulses in a 1 s section in the middle portion of the call) and intercall duration (s) were averaged from 6–10 consecutive calls from each male. Vocal effort was calculated for each individual using the following equation:

\[
\text{Vocal effort (pulses/s)} = \left( \frac{\text{call duration}}{\text{call duration} + \text{intercall duration}} \right) \times \text{pulse rate}
\]

(SPL measures were not acquired in many cases and, therefore, not included in equation (1). Relationships between SPL and other call parameters in the equation

<table>
<thead>
<tr>
<th>Year</th>
<th>April</th>
<th>May</th>
<th>June</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>3–5, 8–9, 13</td>
<td>19</td>
<td>None</td>
</tr>
<tr>
<td>2002</td>
<td>10–17</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2003</td>
<td>12–4, 16–23, 26–27</td>
<td>None</td>
<td>10–12</td>
</tr>
<tr>
<td>2004</td>
<td>None</td>
<td>None</td>
<td>9–11</td>
</tr>
</tbody>
</table>
were thus examined for the subset of individuals for which SPL measures were obtained using simple linear regression. SPL was not significantly correlated with any component of vocal effort \((P > 0.33, N = 149)\), suggesting that SPL did not explain additional variation in vocal effort that was not accounted for in our equation.

Body temperature can alter various vocal attributes in anurans (Gerhardt & Huber 2002; see Brown & Littlejohn 1972 for temperature effects on vocalizations in Bufo). All B. woodhousii in this population called while the hindlegs, pelvis and forelimbs were under water. Calling males maintained cloacal body temperatures that reflected water temperatures (C.J.L. & A.M.G., personal observation). Water temperatures in the near vicinity of calling males were thus used to approximate body temperatures. Vocal effort was corrected to the mean recording temperature (20 °C, SD = 2.9) by calculating residual values obtained from plotting vocal effort against body temperature and using the equation of the regression line (Leary 2001).

**Body Condition**

Relative body condition estimates were calculated by obtaining the residual values from a linear regression of the cubed-root mass on snout–ischial length (SIL) and dividing those values by the SIL following the methods of Baker (1992), Howard et al. (1997) and Howard & Young (1998). This was done for all individuals collectively across all years, allowing values to be compared within and across years. For an 80 mm (SIL), 60.0 g toad, a 0.001 change in body condition was equivalent to approximately a 1.0 g change in mass.

**Hormone Analyses**

We have previously described our hormone extraction, chromatography and radioimmunoassay procedures (Leary et al. 2004), and the methods were validated in this previous study. Briefly, plasma samples were extracted with diethyl ether, dried under nitrogen gas and resuspended in 10% ethyl acetate in iso-octane. Column chromatography was then used to separate dihydrotestosterone (DHT), testosterone (T) and corticosterone (CORT). Antibody for use in the T assay was obtained from Research Diagnostics (Flanders, NJ, U.S.A.) and was also used for measuring DHT because of its cross-reactivity with this hormone. Corticosterone antibody was purchased from ICN Laboratories (Costa Mesa, CA, U.S.A.).

We analysed 368 plasma samples from calling males in six assays. Samples from 2001 were run in several assays containing only that year (see Leary et al. 2004). Samples from 2002–2004 were randomized within assays with respect to time of night sampled, night sampled and year sampled. This approach prevented confounding of assay variability and temporal variation in hormone levels. Mean intra-assay coefficients of variation among the six assays for DHT, T and CORT were 12.9%, 11.5% and 9.2%, respectively, based on four standards run with each assay. Interassay coefficients of variation for DHT, T and CORT were 19%, 16.3% and 10.8%, respectively.

**Statistical Analyses**

**Missing data**

The data set collected had missing data in two forms. First, viewed as a potentially longitudinal study, the data were incomplete in the sense that not every individual measured at least once in a given year was measured again during that year. This was largely attributable to two factors: (1) concerns regarding the physiological effects of repeated sampling (e.g. Moore et al. 1991) and (2) the uncertainty of how many and which individuals would be present on the subsequent night(s) of chorus activity.

Importantly, however, missing data were missing largely by design, and hence uncorrelated with the variables being measured. Apart from concerns regarding loss of statistical efficiency, the usual concerns about otherwise-compromised (i.e. biased) statistical inferences due to missing data are thus not relevant here.

**Dealing with both forms of missing data**

In light of the two forms of missing data noted above, a data management strategy was devised to simultaneously: (a) allow maximal utilization of the information contained in the data and (b) allow a technically correct statistical analysis. This was accomplished via the following steps.

(1) Cases were partitioned according to the missing value pattern of the four dependent variables (androgen level, CORT level, vocal effort, body condition). Only four of the 16 partitions contained substantial numbers of cases and, hence only these were used in (2) and (3) below.

(2) To maximize the information in the cases retained, repeated measures from the same animal within the same year were eliminated from each of the four partitions, retaining only the first-measured case for each animal during a given year.

(3) Available data from each partition was then extracted and appropriately combined across partitions.

We describe the details of this process below.

**Missing data partitions**

The dependent variables were indexed by: 1 = androgen level (DHT + T); 2 = CORT level; 3 = vocal effort; 4 = body condition. For each case in the data, corresponding indicator variables \(i_1, i_2, i_3\) and \(i_4\) were defined by:

\[i_1 = 1\] if androgen level was not missing, \(= 0\) if androgen level was missing;

\[i_2 = 1\] if CORT level was not missing, \(= 0\) if CORT level was missing;

\[i_3 = 1\] if vocal effort was not missing, \(= 0\) if vocal effort was missing;

\[i_4 = 1\] if body condition was not missing, \(= 0\) if body condition was missing.

Then, the 16 distinct values of \((i_1,i_2,i_3,i_4)\) in the set \{\((0,0,0,0), (0,0,0,1), \ldots, (1,1,1,1)\}\) define 16 disjoint subsets, \(d(i_1,i_2,i_3,i_4)\) of the data. For example, \(d(1,1,1,1)\) consisted of the cases for which all the variables were not missing, \(d(1,1,1,0)\) consisted of the cases missing only body condition, and so forth. Thus, the original 985 observations (including all 4 years and all repeated measurements) were...
partitioned according to the missing data patterns shown in Table 2. These subsets were further modified to delete repeated measures of the same animals within a given year, as follows.

1. The subset D1111 was sorted by year, tattoo number (ID) and date, and only the first case with a given year, ID and date was retained (thus eliminating any repeated measurements of the same ID on the same date within the same year).

2. Then D1111 was screened again and only the case for the first date for each ID within a year was retained (thus eliminating any repeated measurements on different dates).

3. Next, D1101 was cleaned by eliminating any records with year and ID appearing in D1111, and then eliminating any repeated measures for other IDs as described in (1) and (2) above.

4. Step 3 was repeated to clean D0011 (relative to the union of D1111 and D1101) and D0001 (relative to the union of D1111, D1101 and D0011).

Thus, our final analysis file consisted of the union of the partitions D1111, D1101, D0011 and D0001, which comprised 593 arguably statistically independent cases (Table 2).

### Justification of deleting repeated measures

Although using repeated measures from the same animals is often an effective strategy for measuring change over time, it becomes much less so in the presence of large amounts of missing data. In our data, all four dependent variables were repeatedly measured on only a very small percentage of the animals, and of these, the intermeasurement time varied considerably (from approximately 1 h to several days). Thus, any proper analysis of the data, accounting for the correlation across repeated measures would also have to account for the intermeasurement times. While such analyses are technically possible, they tend to require relatively large, complete data sets. To attempt such analyses with our small subsample of complete repeated measures would be statistically inefficient. The idea of incorporating into such an analysis some of the partial data from the incomplete data partitions was attempted but, combined with the intermeasurement time issue, was a statistically strained approach (i.e. too many modelling assumptions and insufficient data).

Clearly, however, the data in partitions D1101, D0011 and D0001 should not be discarded, since they certainly contain useful (albeit partial) information. Details of how we incorporated these partial data are provided in the Statistical inference section below.

### Statistical inference

For a single individual, let \( Y = (\ln(A), \sqrt{\text{CORT}}, E, C) \) denote a \( 4 \times 1 \) vector measurement, whose components are, respectively, ln(androgen), square root of corticosterone level, vocal effort and body condition. The transforms of androgen and corticosterone were obtained based on a residual analysis from fitting a flexible day/time trend model to make the components of \( Y \) as close to normally distributed as possible. Our analysis assumed that, for a fixed day and time in the toad chorus, \( Y \) follows a multivariate normal distribution with possibly a day/time-dependent mean vector but a covariance matrix that is day/time invariant.

### Estimating correlations among the dependent variables

Under the aforementioned assumptions, we first used standard methods (see Graybill 1976) to estimate the correlations of the components of \( Y \) from any of the four data partitions that so permitted. Thus, D1111 allowed estimation of all six of the pairwise correlations, D1101 allowed estimation only of the correlations not involving vocal effort, and D0011 allowed estimation only of the correlation between vocal effort and body condition. For correlations estimated in more than one of the partitions, the estimates were combined (under the data-structure-induced assumption that the partition estimates were statistically independent) by standard Fisher variance-weighted \( Z \) transform methods to produce the estimated correlations and 95% confidence intervals shown in Table 3. The hypothesis tests were based directly on the \( Z \) transforms and their asymptotic standard errors (Graybill 1976).

### Estimating time trend functions

Time trend functions were estimated using a segmented regression model as follows. For a given year, let day into the breeding season be indexed by \( d = 1, \ldots, 7 \) with \( d = 1 \) denoting the first day. Within each day, let \( t = 0, 1, \ldots \) denote hour of the day, with \( t = 0 \) denoting 2100 hours. Then, if \( \varepsilon_{idy} \) represents any of the four (possibly transformed) dependent variables measured on individual \( i \) during hour \( t \) of day \( d \) within year \( y \), the model is:

\[
Z_{idy} = b_{0d} + b_{1d} \times t + S_{y} + \varepsilon_{idy} \tag{Model 1}
\]

where:

- \( b_{0d} \) = mean level of \( Z \) at \( 2100 \) hours on day \( d \) (regardless of year)
By using the MIXED procedure in SAS, Model 1 can be estimated simultaneously for all day and time, and a variety of hypotheses addressed as follows.

(1) Whether mean \( Z \) varies over days (i.e., whether the regression slope \( b_{1d} \) differs from zero), and if so, how. We thus test the ‘zero slopes’ hypothesis: \( H_{0ZS} \) if this hypothesis was not rejected at alpha = 0.05, then hypotheses 1–3 were accepted. If this hypothesis was rejected, then we proceeded to test the hypotheses using Scheffe critical values.

Table 3. Trend analysis results for four variables against day (\( d \)) within breeding season for Woodhouse’s toads (fitting Model 1, see Methods)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day within season</th>
<th>Estimate of ( b_{1d} )</th>
<th>Estimate of ( b_{1d} )</th>
<th>( p ) value for ( H_{0SUP} )</th>
<th>( H_{0ES} )</th>
<th>( H_{0EI} )</th>
<th>( H_{0ZS} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln (androgen)</td>
<td>( d=1 )</td>
<td>5.59 (0.18)*</td>
<td>–0.07 (0.06)</td>
<td>0.15</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.26 (0.14)</td>
<td>0.04 (0.04)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.93 (0.14)</td>
<td>0.14 (0.05)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.41 (0.31)</td>
<td>–0.05 (0.06)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.34 (0.32)</td>
<td>–0.01 (0.12)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.49 (0.22)</td>
<td>–0.01 (0.10)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5.15 (0.18)</td>
<td>0.05 (0.08)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>( \sqrt{CORT} )</td>
<td>( d=1 )</td>
<td>6.14 (0.56)</td>
<td>–0.39 (0.20)</td>
<td>&lt;0.0001</td>
<td>Accept</td>
<td>Reject</td>
<td>Reject</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.81 (0.43)</td>
<td>–0.37 (0.14)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.36 (0.44)</td>
<td>–0.29 (0.17)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.20 (0.97)</td>
<td>–0.54 (0.17)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.56 (1.00)</td>
<td>–0.34 (0.37)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.56 (0.69)</td>
<td>0.11 (0.32)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.89 (0.57)</td>
<td>–0.36 (0.23)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vocal effort</td>
<td>( d=1 )</td>
<td>34.8 (2.8)</td>
<td>–1.6 (0.6)</td>
<td>0.001</td>
<td>Accept</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33.9 (2.7)</td>
<td>–1.0 (0.6)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29.5 (3.7)</td>
<td>0.8 (1.4)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>45.6 (1.13)</td>
<td>–7.9 (7.3)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>39.2 (4.6)</td>
<td>–1.4 (1.6)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>32.1 (4.0)</td>
<td>–1.1 (1.8)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>38.0 (3.2)</td>
<td>–1.0 (1.3)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Body condition</td>
<td>( d=1 )</td>
<td>–0.07 (0.51)</td>
<td>0.23 (0.14)</td>
<td>0.0008</td>
<td>Accept</td>
<td>Accept</td>
<td>Accept</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.46 (0.51)</td>
<td>0.00 (0.13)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.00 (0.64)</td>
<td>0.07 (0.22)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–0.60 (1.13)</td>
<td>–0.05 (0.21)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.52 (1.14)</td>
<td>–0.48 (0.37)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.20 (0.83)</td>
<td>–0.18 (0.29)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>–0.78 (0.64)</td>
<td>–0.13 (0.22)</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ES, equal slopes; EI, equal intercepts; SUP, super; ZS, zero slopes (where \( b \) = slope). CORT = corticosterone level.

*Standard errors are in parentheses.

ES, equal slopes; EI, equal intercepts; H0, hypothesis; SUP, super; ZS, zero slopes (where \( b \) = slope). CORT = corticosterone level.

\( b_{1d} \) = slope of assumed linear regression function relating mean \( Z \) to hour, \( t \), on day \( d \) (regardless of year)

\( S_y \) = random effect of year \( y \), assumed to be \( N(0, \sigma_y^2) \), normal distribution with mean 0 and variance year \( y \) data.

\( E_{ydti} \) = random error term representing unmodelled sources of error, assumed to be statistically independent of \( S_y \).

Note that Model 1 posits a linear relationship between \( Z \) and \( t \) on each day, \( d \), but allows the absolute levels and slopes of these relationships to vary over \( d \). Note also that the different years of observation are treated essentially as replications of the same observational conditions specified by \( d \) and \( t \).

Lastly, to maintain control of the type I error rates over the entire family of hypotheses indicated in 1–3 above, the Scheffe procedure was applied to each dependent variable separately. Specifically, an a priori superhypothesis, \( H_{0SUP} \), whose truth implies that of all the hypotheses in 1–3, was tested first. \( H_{0SUP} \) consists of the assertion that both \( H_{0EI} \) and \( H_{0ZS} \) hold simultaneously.

If this hypothesis was not rejected at alpha = 0.05, then hypotheses 1–3 were accepted. If this hypothesis was rejected, then we proceeded to test the hypotheses using Scheffe critical values.

Androgen—vocal effort analysis using repeated measures

The EHV model predicts dose-dependent relationships between androgen levels and vocal effort. To further test these predictions, we took advantage of the repeated measures data that were deleted from the previous analyses. This approach has the advantage of helping to control for, or at least minimizing, potential variation in hormone-binding proteins, receptor levels and/or hormone threshold levels that may exist among individuals and, hence, mask correlations between the measured variables in the aforementioned statistical model. Although the EHV model also predicts that corticosterone level and vocal effort will be positively correlated, this
Temporal Patterns

Androgen levels did not change significantly within or across nights of chorus activity (trend analysis using Model 1, \( N = 293, P = 0.15 \) for \( H_0^{SUP} \); Table 3, Fig. 1a). Although there were apparently considerable changes in mean androgen levels within nights of chorus activity, the standard errors of the slope estimates were large, so there were no detectable nonzero slopes (Table 3, Fig. 1a).

For corticosterone levels, \( H_0^{SUP} \) was strongly rejected (trend analysis using Model 1, \( N = 293, P < 0.0001 \), \( H_0^{S} \) was rejected, \( H_0^{E} \) was rejected and \( H_0^{I} \) was accepted (Table 3). The interpretation of these results is that there was the same statistically significant decline in corticosterone levels within each day of the breeding season, but the absolute corticosterone levels tended to vary across days (specifically, they tended to decrease across nights of breeding activity) (Table 3, Fig. 1b).

For vocal effort, \( H_0^{SUP} \) was rejected (trend analysis using Model 1, \( N = 246, P = 0.001; \) Table 3), but strict adherence to the Scheffe decision procedure resulted in the counter-intuitive (but common) decision that neither \( H_0^{ZS} \), \( H_0^{ES} \) nor \( H_0^{I} \) was rejected. However, the marginal \( P \) value for \( H_0^{ZS} \) was 0.06, suggesting a nearly significant result for this hypothesis. The interpretation of these results is that there was little evidence of any time trend of vocal effort, and possibly some nonzero (but otherwise nondescript) time slopes across the days (Fig. 1c).

For body condition, \( H_0^{SUP} \) was rejected (trend analysis using Model 1, \( N = 593, P = 0.0008; \) Table 3), but again adherence to the Scheffe decision procedure resulted in none of the other hypotheses (\( H_0^{ZS} \), \( H_0^{ES} \) or \( H_0^{I} \)) being rejected. None of the marginal \( P \) values for these three subhypotheses was smaller than 0.45, so there was no evidence against any of them. The interpretation of these results is that there was no evidence of detectable change in body condition within or across nights of chorus activity (Table 3, Fig. 1d).

Correlation Analysis of Relationships Among Variables

Estimates of all possible pairwise correlations among the four measured variables are reported in Table 4. The marginal (i.e. one at a time) \( P \) values are shown for the null hypothesis that the population correlation is zero. When applying the Bonferroni procedure for controlling the family-wise type I error probability over the six tests, the resulting \( P \) value had to be below 0.05/6 = 0.008 to be considered significant.

Interpreted under strict Bonferroni control, the results indicated that only androgen level and corticosterone level were significantly, and positively, correlated (\( P < 0.0001; \) Table 4).

If such strict concern over type I errors is relaxed, androgen level and body condition, as well as corticosterone level and vocal effort, were also highly and positively correlated (\( P = 0.02; \) Table 4). The remaining pairwise correlation estimates did not differ significantly from zero (\( P > 0.44; \) Table 4).

Repeated Measures

Further analysis of the androgen level–vocal effort relationship using repeated measures data indicated that vocal effort was not significantly positively correlated with androgen level. Specifically, although we observed a mean \( \pm SE \) difference across days in circulating androgen levels of 117.52 \( \pm 31.31 \) ng/ml, the regression \( F \) test of \( H_0 \) (\( b_1 = 0 \) in Model 2) was \( P = 0.68 \).

DISCUSSION

There is considerable evidence that acoustic signals can modulate the activity of reproductive neuroendocrine brain regions in various animals including fish (Remage-Healey & Bass 2004, 2005), amphibians (Wilczynski & Allison 1989;
Burmeister & Wilczynski 2005), mammals (McComb 1987) and birds (Hinde & Steele 1978; Cheng & Zuo 1994; Cheng et al. 1998). Furthermore, stimulatory effects of conspecific, but not heterospecific, calls on gonadal development and/or androgen levels have been documented in numerous anuran species (Brzoska & Obert 1980; Chu & Wilczynski 2001; Lea et al. 2001; Burmeister & Wilczynski 2005). Hence, it is surprising that androgen levels in calling male *B. woodhousii* did not vary temporally in a manner consistent with the EHV model. For instance, there was no evidence that androgen levels increased within or across nights of breeding activity or that androgens were low at the onset of yearly chorus activity or towards the end of the breeding period. Rather, mean androgen levels remained relatively unchanged within and across nights of chorus activity. One possible explanation for the observed pattern is that environmental cues such as rainfall rapidly activate the HPG axis in this species, creating a ceiling effect prior to the formation of the chorus and the onset of vocal activity (yearly chorus activity was, in all cases, initiated by heavy rainfall as were most nights of chorus activity). This hypothesis is consistent with hormone–behaviour relationships that are expected to occur in explosive-breeding species that live in unpredictable environments and mate opportunistically (Crews & Moore 1986). If androgen production is stimulated by rainfall and androgen levels are well above those required for breeding activity, hearing and/or producing calls may have little additional effect on circulating gonadal steroid levels in Woodhouse’s toads.

Androgen levels also did not accurately predict changes in vocal effort in Woodhouse’s toads. Although there is evidence of dose-dependent relationships between androgens and sexually selected male traits (as predicted by the EHV model), such hormone–behaviour relationships are uncommon (reviewed by Hewes & Moore 1997; Adkins-Regan 2005). One possibility, consistent with the hypothesis that environmental cues create a ceiling effect with respect to circulating androgen levels, is that the range of androgen levels in natural choruses of Woodhouse’s toads are above threshold levels required to elicit maximum vocal effort. Hence, further elevations above threshold may have little or no effect on vocal effort (see review by Adkins-Regan 2005). Under these conditions, call characteristics may be

**Table 4.** Correlation results for all pairwise comparisons among androgen levels, corticosterone (CORT) levels, vocal effort and body condition in Woodhouse’s toads

<table>
<thead>
<tr>
<th>Variables in correlation</th>
<th>Correlation</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln (androgen level) and √CORT</td>
<td>0.22</td>
<td>&lt;0.0001</td>
<td>0.093, 0.336</td>
</tr>
<tr>
<td>ln (androgen level) and vocal effort</td>
<td>0.03</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>ln (androgen level) and body condition</td>
<td>0.15</td>
<td>0.02</td>
<td>0.027, 0.275</td>
</tr>
<tr>
<td>√CORT and vocal effort</td>
<td>0.20</td>
<td>0.02</td>
<td>0.021, 0.367</td>
</tr>
<tr>
<td>√CORT and body condition</td>
<td>0.06</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Vocal effort and body condition</td>
<td>0.06</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Model-smoothed mean (a) androgen levels, (b) corticosterone levels, (c) vocal effort and (d) body condition within and across nights of chorus activity in Woodhouse’s toads. Hours within nights are indicated by small tick marks on the abscissa (beginning at 2100 hours). Whiskers represent Bonferroni simultaneous 95% confidence intervals. See Methods for details.
modulated by social interactions, but vocal changes do not track fluctuations in androgen levels. Androgen manipulation studies that address these issues have not been performed in any anuran species. Such studies, analogous to the extensive work done in dark-eyed juncos by Ketterson et al. (2001), are needed for this group to further understand the relationships among androgens and vocal characteristics (Leary et al. 2006b).

A key prediction of the EHV model is that an increase in circulating levels of corticosterone in calling males should be detectable over multiple temporal scales, including within nights as well as across nights of chorus activity. Nightly elevation of corticosterone levels was predicted to result from the depletion of energy reserves associated with temporal elevations in androgen levels and vocal effort. Corticosterone levels were also expected to increase across nights of chorus activity because the rate of nightly corticosterone production was predicted to exceed the rate of corticosterone metabolism during periods of daytime inactivity when corticosterone levels should be decreasing (Emerson 2001). In contrast to these predictions, we found that corticosterone levels in calling Woodhouse’s toads tended to decrease within and across nights of chorus activity. Furthermore, because corticosterone levels were generally highest at the onset of nightly chorus activity, our results suggest that corticosterone levels actually increased during periods of daytime inactivity.

The EHV model predicts that nightly elevations in corticosterone will be driven by the depletion of energy reserves associated with temporal elevations in androgen levels and vocal effort. Hence, observed temporal changes in corticosterone levels in calling male Woodhouse’s toads may have been inconsistent with the model because there was little support for underlying predictions that would mediate such a response (i.e. there was no evidence that androgens or vocal effort increased temporally). However, our results suggest that energy—corticosterone—vocal effort relationships may be more complex than predicted. For example, although corticosterone was significantly correlated with vocal effort (as predicted by the EHV model), it did not show an inverse correlation with body condition. These results suggest that local environmental and/or social factors that promote increased vocal effort may also elicit increased corticosterone levels independently of available energy reserves or androgen levels. One possibility that we are currently investigating is whether variation in chorus density could explain such patterns. For example, overcrowding associated with high population density increases corticosterone levels in European starlings, *Sturnus vulgaris*, and such effects may occur independently of food availability or metabolic state (Nephew & Romero 2003). Elevations in vocal effort associated with high chorus density in some anurans (Wells & Taigen 1986) lend further support for this hypothesis.

Corticosterone levels can be influenced by a variety of intrinsic and extrinsic factors (reviewed by Wingfield & Sapolsky 2003) that could contribute to the temporal patterns that we observed in Woodhouse’s toads. For example, as with androgen levels, local environmental conditions may also stimulate the production of corticosterone; yearly and nightly initiation of chorus activity was typically associated with heavy rainfall when corticosterone levels were highest. These results may provide evidence that high corticosterone levels at the onset of vocal activity help to prepare the organism for future stressful circumstances (concepts of the ‘preparatory hypothesis’; Sapolsky et al. 2000; Romero 2002).

Alternatively, the presence of potential predators also may have influenced nightly changes in corticosterone levels. Various potential predators occurred at the chorus site and, in some cases, were directly observed to prey on adult Woodhouse’s toads (e.g. raccoons, *Procyon lotor*, skunks, *Mephitis mephitis*, and water snakes, *Nerodia fasciata*; C.J.L. & A.M.G., personal observation). Such predators were observed only rarely and were typically found during periods when toads were migrating to the pond. We usually arrived at the site before chorus activity began (at dusk), and predators typically fled as we approached the pond. Hence, predator effects may account for the high corticosterone levels early in the evening when toads were migrating to the pond. However, there was no evidence that predators affected seasonal changes in corticosterone levels by differential abundance across the breeding season. Given that corticosterone levels tended to decrease within nights of calling activity, we assume that our presence at the chorus did not alter temporal changes in corticosterone. We have previously addressed the potential effects of handling, measuring and blood sampling on corticosterone levels in this species (Leary et al. 2006a) and found no evidence that our presence or procedures elicited elevations in corticosterone levels. In addition, calling males rarely stopped vocalizing in response to our approach (e.g. when obtaining vocal recordings), suggesting that it is unlikely that we are perceived as a threat.

Importantly, observed temporal patterns for steroid hormone levels did not appear to be related to immigration of new individuals into the chorus. This species breeds explosively and only marked males were typically found at the chorus over the course of the breeding season (C.J.L. & A.M.G., unpublished data). It is also unlikely that the lack of a significant increase in corticosterone levels over time reflects low energetic costs of vocal production in this species. Woodhouse’s toads actively modulate the amplitude of their calls, forming pulse trains by rapidly contracting the thoracic musculature throughout the duration of the call (unlike anurans that passively modulate the amplitude of their calls; see review of bufonids: Martin 1972). Active modulation is expected to require more energy per unit time than passive modulation (reviewed by Wells 2001).

Although glucocorticoids play a well-known role in energy metabolism and are expected to be elevated during periods when the organism is in a state of negative energy balance (Sapolsky 1992a, b; Laugero 2001; McEwen & Wingfield 2003), we found little evidence to support the prediction that corticosterone levels were negatively correlated with body condition in Woodhouse’s toads. These
results may be attributable to the various intrinsic and extrinsic factors that potentially modulate corticosterone levels and mask the predicted relationship. We also acknowledge that there are potential problems associated with body condition indexes (the proxy for energetic state) used in the current study. Without verification that condition indexes accurately reflect energy reserves, we cannot be confident that nonsignificant relationships between corticosterone and body condition accurately depict relationships between corticosterone and energy stores. For example, the increase in body condition on the first night of calling activity may have been indicative of increased foraging during this period or, alternatively, water uptake upon arrival to the pond following a prolonged period of hibernation. Nevertheless, high body condition indexes were associated with high androgen levels, suggesting that energetic state may be an important determinant of androgen levels (reviewed by Adkins-Regan 2005).

Emerson (2001) indicated that males of certain anuran species may have feeding patterns that yield inconsistencies with various components of the EHV model. For example, in species that regularly feed at the chorus site, males may not experience elevated levels of corticosterone that initiate a stress response (i.e. a decrease in androgen levels). Woodhouse's toads do not feed at the breeding pond (Woodward 1982) but have been observed to leave the chorus, forage in nearby areas, and subsequently return to the chorus (C.J.L. & A.M.G., personal observation). Such feeding patterns may be frequent enough to maintain similar body condition indexes within and across nights of breeding activity in this species.

The current study provides little support for the EHV model. We propose that inconsistencies with the model are primarily related to presumed dose-dependent relationships between hormone levels and vocal effort, and the variety of intrinsic and extrinsic factors that potentially modulate circulating hormone levels. Variation in hormone-binding proteins and/or receptor levels may further complicate the predicted relationships. These factors may yield inconsistencies with model predictions or present some difficulty in tracking the expected changes in naturally occurring anuran choruses. Our study is the only investigation that has concurrently examined temporal patterns and relationships among androgen levels, corticosterone levels, vocal effort and body condition in an anuran. Whether the model accurately describes temporal patterns and relationships among these factors in other anuran species is not known but warrants further comparative studies.

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