



Close-range vocal signals elicit a stress response in male green treefrogs: resolution of an androgen-based conflict



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Male courtship signals often stimulate the production of sex steroids in both female and male receivers. Such effects benefit signallers by increasing receptivity in females, but impose costs on signallers by promoting sexual behaviour and aggression in male competitors. To resolve this androgen-based conflict, males should use strategies that suppress sex steroid production in rival males. In green treefrogs, *Hyla cinerea*, chorus sounds (i.e. advertisement calls from aggregates of males) are known to stimulate androgen production in receiver males. Here, I examined whether males of this species counter these effects by eliciting an endocrine stress response in male conspecifics during close-range vocal interactions. I show that corticosterone (CORT) levels were higher in males that lost vocal contests in natural choruses compared to contest winners and nonaggressive males. Testosterone levels were also lower in contest losers compared to nonaggressive males, but not contest winners; dihydrotestosterone levels did not differ among the three groups. Aggressive and advertisement calls were then broadcast to males in an experiment that simulated close-range vocal communication. Aggressive calls rapidly (45 min) elicited an increase in CORT and a reduction in androgens in receivers. Advertisement calls did not elicit an increase in CORT, but CORT levels were sustained relative to controls exposed to silence and were accompanied by a reduction in androgens in small males. Endocrine responses to acoustic signals in this species thus vary depending upon context, call type and size of signal receivers. Signallers benefit from eliciting CORT production in competitors because elevated CORT suppresses vocalization.

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Male courtship signals characteristically increase receptivity in conspecific female signal receivers by stimulating the production of sex steroids (Adkins-Regan, 2005; Lynch & Wilczynski, 2006; Maney, Goode, Lake, Lange, & O'Brien, 2007; Marshall, 1936; Nelson, 2011; Propper & Moore, 1991; Remage-Healey & Bass, 2004; Wingfield & Marler, 1988). Male signals presumably vary in the extent to which they trigger neuroendocrine cascades, thus providing a hormonal basis for female mate preferences that can drive the evolution of male sexual signals (Andersson, 1994; Marshall, 1936). A conflict arises, however, when male sexual signals also stimulate the production of sex steroids in rival conspecific male receivers. Such circumstances actually appear to be common and widespread across vertebrate taxa. For instance, social interactions involving the exchange of courtship signals characteristically elevate circulating androgen levels above baseline levels in male birds (e.g. concepts of the 'challenge hypothesis': Wingfield, Ball, Dufty, Hegner, & Ramenofsky, 1987; Wingfield

et al., 1999; Wingfield, Hegner, Dufty, & Ball, 1990) and many other vertebrate taxa as well (Hirschenhauser, Taborsky, Oliveira, Canario, & Oliveira, 2004; reviewed in: Hirschenhauser & Oliveira, 2006; Oliveira, Hirschenhauser, Carneiro, & Canario, 2002; Soma, 2006). While such a response promotes reproductive behaviour and/or aggression in males interacting with sexually receptive females and/or competing with other males that benefit signal receivers (Wingfield et al., 1987, 1990, 1999), there has been little emphasis on the problems such an effect poses for signal senders. Androgen-mediated effects on mating behaviour and aggression in rival males, for example, are likely to impose costs on signalling males. Stimulatory effects of male sexual signals on androgen production are particularly problematic for signal senders if androgen level is related to the magnitude of sexually selected traits that are preferred by females (i.e. see Emerson, 2001; Folstad & Karter, 1992). Males are thus expected to use strategies that counter the effects of courtship signals on androgen production in rival males.

As with many other organisms, the acoustic courtship signals (i.e. advertisement calls) of anuran amphibians (frogs and toads) stimulate neuroendocrine cascades that induce sexual readiness and coordinate reproductive activity between the sexes (Burmeister

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& Wilczynski, 2000; Chu & Wilczynski, 2001; Wilczynski, Allison, & Marler, 1993; Wilczynski & Chu, 2001; Wilczynski & Lynch, 2011; Wilczynski, Lynch, & O'Bryant, 2005). Exposure to conspecific male advertisement calls, for example, increases the activity of hypothalamic neurons (Allison, 1992; Wilczynski & Allison, 1989) and stimulates oestradiol production, ovarian development and receptivity in females (Lea, Dyson, & Halliday, 2001; Lynch & Wilczynski, 2006; Rabb, 1973; Wilczynski & Lynch, 2011). Conspecific advertisement calls also stimulate testicular development and/or androgen production in males (Brzoska & Obert, 1980; Burmeister & Wilczynski, 2000, 2005; Chu & Wilczynski, 2001), consistent with predictions of the challenge hypothesis (Emerson, 2001; Wingfield et al., 1990). Female and male anurans thus appear to share similar neural pathways that are stimulated by the same acoustic signals and that modulate sex steroid production.

Here, I examined how close-range aggressive vocal signals influence the endocrine physiology of male green treefrogs, *Hyla cinerea*. Males of this species produce two main types of acoustic signals during close-range aggressive vocal interactions: advertisement calls and aggressive calls (Gerhardt, 1978a; Oldham & Gerhardt, 1975). Advertisement calls have a dual function in that they are used to attract females and/or ward off rival males, whereas aggressive calls are produced almost exclusively in the context of male–male close-range interactions (reviewed in: Gerhardt & Huber, 2002; Wells, 2007). Detection of nearby conspecific males typically results in the production of advertisement calls that are directed at the encroaching individual, but interactions may escalate to include the production of aggressive calls (reviewed in Gerhardt & Huber, 2002; see also Reichert, 2011; Reichert & Gerhardt, 2013). The two calls differ primarily in the amplitude time envelope (Gerhardt, 1978a). For example, advertisement calls are largely unpulsed (i.e. are not amplitude modulated) with the exception of a short pulsed prefix that is characteristically present at the onset of the call (Gerhardt, 1978b, 1981; see Fig. 1a). In contrast, aggressive calls are highly pulsed throughout the duration of the call (Gerhardt, 1978b, 1981; see Fig. 1b).

Hyla cinerea was used in the current study because previous work has shown that chorus sounds (i.e. broadcast recordings of advertisement calls from aggregates of calling males) stimulate the production of androgens in male signal receivers (Burmeister & Wilczynski, 2000, 2005). However, a different hormonal response appears to emerge during close-range vocal interactions (which

occur frequently in this species, especially in dense choruses). For example, males that lose close-range vocal contests and adopt an alternative noncalling 'satellite' mating tactic (see also Reichert & Gerhardt, 2014, for evidence in grey treefrogs, *Hyla versicolor* and *Hyla chrysoscelis*) have higher glucocorticoid levels and lower androgen levels than calling males (Leary & Harris, 2013). Circulating glucocorticoids and testosterone (but not dihydrotestosterone) are inversely related in this species (Leary & Harris, 2013), suggesting that close-range vocal interactions elicit a stress response in these males. However, Leary and Harris (2013) did not assess the extent to which differences in circulating hormone levels among males were related to differences in body condition (a proxy for energy reserves) or body size. Satellite males, for example, are typically in poorer body condition and smaller than calling males. Body condition is inversely related to glucocorticoid level and positively correlated with androgen level in this species (Leary & Harris, 2013), suggesting that high glucocorticoids and low androgens in satellite males may be attributable to poor body condition. Alternatively, differences in hormone levels in satellite and calling males may reflect size-related variation in the endocrine responses of males to close-range vocal interactions.

Here, I expand upon this previous work on *H. cinerea* by comparing circulating hormone levels in naturally occurring contest winners and losers. Moreover, I examine the endocrine responses of males to broadcast advertisement calls and aggressive calls using an experimental playback paradigm that simulated close-range vocal interactions. Lastly, I assess how both body condition and body size contribute to variation in circulating hormone levels of signal receivers. There is an extensive literature on the effects of winning and losing contests on circulating glucocorticoid and androgen levels. However, there is surprisingly little conformity in the endocrine responses among species (Creel, 2001; Creel, Dantzer, Goymann, & Rubenstein, 2013; Fuxjager & Marler, 2010; Gleason, Fuxjager, Oyegbile, & Marler, 2009; Hsu, Earley, & Wolf, 2006), making it difficult to predict how the outcome of aggressive contests potentially influences circulating hormone levels. For example, whether dominant or subordinate males have higher circulating glucocorticoid levels may depend on prior experiences, the stability of the social hierarchy and/or environmental conditions (i.e. food availability and predation risk; Creel, 2001; Creel et al., 2013; Hsu et al., 2006). Nevertheless, previous work in *H. cinerea* (Leary & Harris, 2013) suggests that contest losers have higher glucocorticoid and lower androgen levels than contest winners and that the magnitude of the endocrine response to close-range vocal interactions is greater for small males.

The impetus for the current study was rooted in the premise that stimulation of androgen production in male competitors is disadvantageous to signal producers, and thus, that signalling males should use strategies that alter the endocrine physiology of receivers so that they gain an advantage over their vocal competitors. Males could achieve this if close-range aggressive signals stimulate the production of glucocorticoids. It is well known that glucocorticoids negatively affect the production of sex steroids via suppression of the hypothalamic–pituitary–gonadal axis, by inhibition of enzymes involved in the synthesis of androgens and/or by stimulation of gonadotropin inhibitory hormone (Calisi, Rizzo, & Bentley, 2008; Chand & Lovejoy, 2011; Greenberg & Wingfield, 1987; Kirby, Geraghty, Ubuka, Bentley, & Kaufer, 2009; Leary & Knapp, 2014; Michael & Cooke, 1994; Monder, Sakai, Miroff, Blanchard, & Blanchard, 1994; Sapolsky, 1992; Wingfield & Sapolsky, 2003). Elevated levels of glucocorticoids have been shown to decrease the quality of vocalization (Leary, Garcia, & Knapp, 2006a) and/or suppress calling behaviour in anurans (Leary, Garcia, & Knapp, 2006b; reviewed by Leary, 2009) including *H. cinerea* (Burmeister, Somes, & Wilczynski, 2001). Previous

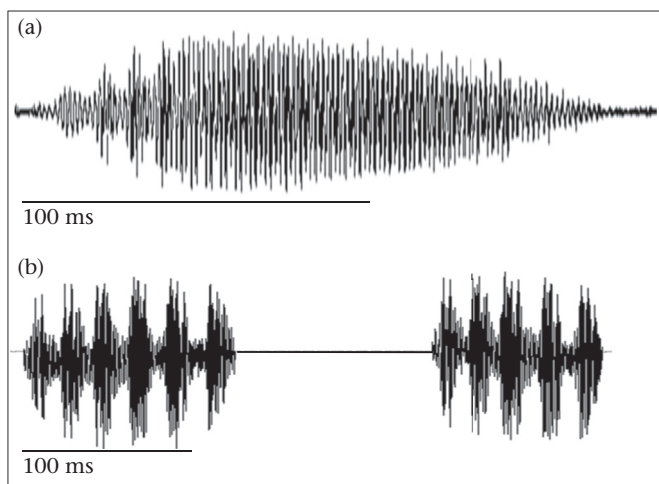


Figure 1. Waveform representations of (a) advertisement and (b) aggressive calls from *Hyla cinerea*.

studies in anurans have focused on agonistic interactions in the context of behavioural responses of receivers (reviewed by: Gerhardt & Huber, 2002; Reichert, 2011; Wells, 2007), but none have examined how close-range aggressive signals affect hormonal responses in signal receivers. I predicted that close-range aggressive calls stimulate the production of glucocorticoids and suppress androgen production in male signal receivers and that this response is most pronounced in small males that typically lose vocal contests. In contrast, close-range advertisement calls were expected to be relatively ineffective in eliciting a stress response in male receivers.

METHODS

Hormone Levels of Males in Natural Choruses

Male *H. cinerea* were observed in natural choruses during the 2010 and 2011 breeding seasons (~May–July) at the University of Mississippi Field Station (Lafayette County, MS, U.S.A.). I obtained blood samples from males that had not been observed to interact in close-range vocal interactions ('nonaggressive males') and from males that had been observed to win ('aggressive winners') or lose ('aggressive losers') vocal contests. Subjects were designated as aggressive losers if they clearly retreated or stopped vocalizing (e.g. adopted a satellite mating tactic) in response to the other male of the interacting pair (i.e. the aggressive winner). All such interactions occurred naturally (i.e. no interactions were staged). I often detected such interactions in natural choruses by listening for aggressive calls and rapidly locating the interacting pair. Hence, for many observations, there was no information regarding previous behaviour of individuals. For instance, satellite males often reside in close proximity to calling males for prolonged periods but periodically challenge nearby calling 'host' males by producing advertisement calls and aggressive calls that are directed at the host male; in many instances, it was not known whether males were behaving as callers or satellites prior to close-range vocal interactions. Similarly, designation of intruder versus resident males often could not be reliably ascertained. Nevertheless, close-range vocal interactions often continued after the interacting pair was located, and all cases involved the exchange of both advertisement calls and aggressive calls between males (e.g. both males in the interacting pair produced advertisement and aggressive calls). Focal observations lasted 15–80 min, with the longest focal periods generally occurring for control males that were observed for at least 30 min to ensure that they had not recently interacted with other males.

Following focal observations (i.e. 5–10 min after a behavioural outcome was established), I captured individuals by hand and obtained blood samples (75–100 μ l) within 1 min via cardiac puncture using a sterile 27-gauge heparinized hypodermic needle. Individuals were then measured from the tip of the snout to the end of the ischium, weighed, marked with a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, U.S.A.) to avoid resampling the same individual, and released at the site of capture. Blood samples were stored on ice and subsequently centrifuged at 2200 revolutions/min upon return to the laboratory. Plasma was stored at -20°C until corticosterone (CORT), dihydrotestosterone (DHT) and testosterone (T) levels were quantified (described below). Data were acquired from 31 nonaggressive males, 32 aggressive winners and 22 aggressive losers. Most contest losers (86%) represented a class of males that adopted an alternative noncalling satellite mating tactic after losing vocal contests with other males (see Leary & Harris, 2013).

Because satellite males are generally in poorer body condition than calling males, and body condition is inversely related to CORT

level and positively correlated with androgen level (DHT + T) in this species (Leary & Harris, 2013), I examined body condition estimates for nonaggressive males, aggressive winners and aggressive losers before comparing circulating hormone levels among the three groups. Body condition was calculated by obtaining the residual values from a linear regression of the cubed-root body mass (g) on snout–ischial length (SIL) and dividing those values by the SIL for all groups of males combined (Baker, 1992; Leary & Harris, 2013; Leary, Jessop, Garcia, & Knapp, 2004). Estimates were then used to adjust circulating hormone levels statistically to the mean body condition value for all groups combined (mean = 0.0001) using the equation of the line for the pooled slope between hormone level and body condition (see Tomkins & Simmons, 2002). Hormone levels adjusted for body condition were then compared among nonaggressive males, aggressive winners, and aggressive losers, and contrasted with results for hormone values that were not adjusted for body condition using ANOVA followed by Tukey–Kramer post hoc tests. Body condition and body size (SIL) for the three groups of males were compared using the same statistical procedures.

Effects of Broadcast Close-range Vocalizations on Hormone Levels

Although potentially informative, examination of circulating hormone levels of males engaged in vocal interactions in natural choruses is limited in its capacity to establish causation. Hence, I performed experiments in the laboratory wherein isolated vocal signals were broadcast from speakers to captive males at a distance of 1–2 m to assess the effects on the endocrine physiology of signal receivers (this is a conservative distance because males in natural choruses will often exchange vocalizations when much closer). I collected calling males from natural choruses during the breeding season in 2012 and placed them in 38-litre aquaria lined with acoustic foam and fitted with a mesh lid. Subjects were fed crickets ad libitum and held in captivity for about 24 h before playback experiments were initiated (experiments took place on the following night between 2100 and 2300 hours, coinciding with periods of peak chorus activity in natural choruses). Experiments were performed on groups of three to six captive individuals until data were acquired from a sufficient number of individuals for statistical analysis. Treatment groups were randomized across the period in which playback experiments were performed to control for potential temporal effects. I compared the effects of broadcast aggressive calls and advertisement calls on the endocrine physiology of signal receivers and control subjects that were treated in the same fashion but exposed to silence.

I used natural calls (isolated from individual males) as stimuli in playback experiments because it is difficult to synthesize calls that contain all the various features of natural calls that may be important in modulating circulating hormone levels in signal receivers. The advertisement call stimulus used in playback experiments (shown in Fig. 1a) consisted of a single isolated advertisement call recorded from an individual possessing peak carrier frequencies of 800 and 2600 Hz that approximated the mean values for the study population (mean low peak carrier frequency = 788 Hz, range 640–1030, $N = 117$; mean high peak carrier frequency = 2550 Hz, range 1724–3600 Hz, $N = 117$). Call duration (189 ms) and intercall interval (612 ms) of the advertisement call stimulus also fell well within the range of natural calls for the study population (mean call duration = 145 ms, range 86–221 ms, $N = 117$; mean intercall interval = 456, range 255–800 ms, $N = 117$). The population intercall interval values were based on consecutive calls produced during a single respiratory cycle and thus do not include the duration between successive bouts of calling, which would increase this measure considerably.

I repeatedly broadcast the call to groups of captive males for 45 min from a SME-AFS Amplified Field Speaker (Saul Mineroff Electronics, Inc., Elmont, NY, U.S.A.) mounted on a tripod at an amplitude of 90 dB SPL (measured with a hand-held sound pressure level meter, Ivie Technologies Inc., model IE-45, fast weighting setting) that approximated the amplitude of natural calls for this species (Humfeld, 2013). Similarly, I selected two consecutive exemplar aggressive calls (durations = 120 ms and 100 ms; see Fig. 1b) from recordings of an individual that produced aggressive calls of similar peak frequencies as the advertisement call stimulus (within 60 Hz). Aggressive calls were digitally manipulated (using Raven software, Cornell Bioacoustics Laboratory, Ithaca, NY, U.S.A. <http://www.birds.cornell.edu/brp/raven/ravenversions>) so that the two exemplar calls were repeated in rapid succession to create a series of four consecutive calls (each separated by 120 ms). Each group of four consecutive calls was separated by 1705 ms and repeatedly broadcast at 90 dB SPL in the same fashion in which advertisement calls were broadcast to experimental subjects.

Blood samples were rapidly (~2 min) acquired from subjects via cardiac puncture immediately before and after stimuli were broadcast to captive males (i.e. 45 min later) or after an equal period of silence for controls. Hence, two blood samples were obtained from all experimental subjects. Circulating hormone levels (CORT, DHT and T) were measured from 18 males exposed to isolated aggressive calls, 18 males exposed to isolated advertisement calls and 16 males exposed to silence.

I compared the effects of broadcast calls and silence on pre- and post-treatment hormone levels using repeated measures ANOVA followed by paired *t* tests. Size-associated variation in hormonal responses to broadcast calls was examined using linear regression analysis, where the magnitude of the hormonal response to broadcast calls was plotted against SIL. The magnitude of the hormonal response to broadcast calls was calculated by subtracting pre-treatment hormone levels from post-treatment hormone levels for each individual.

Column Chromatography and Radioimmunoassay Procedures

Hormone separation and quantification of hormone concentrations followed the protocol described in Leary and Harris (2013). Briefly, plasma samples were incubated overnight with radio-labelled hormone (PerkinElmer, Inc. Hebron, KY, U.S.A.) 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. T antibody was obtained from Fitzgerald Industries International, Inc (Acton, MA, U.S.A.) and used for both T and DHT assays. CORT antibody was purchased from MP Bio-medicals, LLC (Solon, OH, U.S.A.). All samples were assayed in duplicate.

Plasma samples from males in natural choruses were analysed for DHT, T and CORT levels in four assays. Mean intra-assay coefficients of variation for DHT, T and CORT were 14%, 9% and 10%, respectively, based on three to four standards run with each assay. Interassay coefficients of variation for DHT, T and CORT were 19%, 18% and 19%, respectively (see Leary & Harris, 2013). Plasma samples from captive subjects used in simulated close-range vocal playback studies were analysed in three assays. All blood samples from interacting males in natural choruses were run in the same

assay as were repeated measures from males from experimental playback experiments. Mean intra-assay coefficients of variation for DHT, T and CORT were 5%, 14% and 4%, respectively, based on three to four standards run with each assay. Interassay coefficients of variation for DHT, T and CORT were 20%, 3% and 10%, respectively. Assay sensitivities were approximately 0.13 ng/ml for DHT, 0.12 ng/ml for T and 0.45 ng/ml for CORT.

Ethical Note

Blood collection procedures largely followed the standard operating procedures outlined by the U.S. Geological Survey National Wildlife Health Center (Madison, WI, U.S.A.). However, an anaesthetic was not used because this procedure can alter circulating hormone levels and exposes the animals to unnecessary stressors. There were no overt effects of the bleeding procedure on experimental subjects (e.g. calling male frogs typically resumed calling and/or mating behaviour within 2 min after release), and all procedures were observed and approved by a veterinarian. Results from the present study indicated that repeated acquisition of blood samples did not cause any detectable increase in circulating stress hormone levels (see Results, for control animals in playback experiment). Scientific collecting permits were provided by the Mississippi Department of Wildlife, Fisheries and Parks (permits 0406111, 0321133, 0326141). All procedures were approved by the University of Mississippi Animal Care and Use Committee (protocols 10-027, 11-021 and 12-02).

RESULTS

Males in Natural Choruses

Nonaggressive males, aggressive winners and aggressive losers differed in body size (i.e. snout–ischial length) (ANOVA: $F_{2,82} = 20.87$, $P < 0.0001$). Contest losers were smaller (mean \pm SE = 49 ± 1.04 mm) than nonaggressive males (55 ± 0.84 mm) and aggressive winners (56 ± 0.46 mm; Tukey–Kramer post hoc tests: $P < 0.05$), but nonaggressive males and aggressive winners did not differ in size ($P > 0.05$), indicating that the aggressive losers were the smallest individuals.

Results indicated that nonaggressive males, aggressive winners and aggressive losers differed in body condition (ANOVA: $F_{2,82} = 4.14$, $P = 0.02$). Aggressive losers were in poorer condition (mean \pm SE = $-0.001 \pm 3.01E-4$) than nonaggressive males ($3.03E-4 \pm 3.16E-4$) (Tukey–Kramer post hoc test: $P < 0.05$), but aggressive winners ($-8.97E-5 \pm 2.55E-4$) and nonaggressive males did not differ in body condition nor did aggressive winners and losers ($P > 0.05$).

CORT levels

There were differences between the three groups of males with respect to circulating CORT levels adjusted for body condition (ANOVA: $F_{2,82} = 9.25$, $P = 0.0002$). Aggressive losers had significantly higher levels of circulating CORT (adjusted for body condition) than both nonaggressive males and aggressive winners (Tukey–Kramer post hoc test: $P < 0.05$), but CORT levels for nonaggressive males and aggressive winners did not differ ($P > 0.05$; Fig. 2a). This same pattern held for CORT values that were not adjusted for body condition (ANOVA: $F_{2,82} = 13.29$, $P < 0.0001$, followed by Tukey–Kramer post hoc tests), indicating that variation in body condition did not explain differences in circulating CORT levels between groups (Fig. 2a, b).

Androgen levels

There were no differences between the three groups of males with respect to circulating DHT levels adjusted for body condition

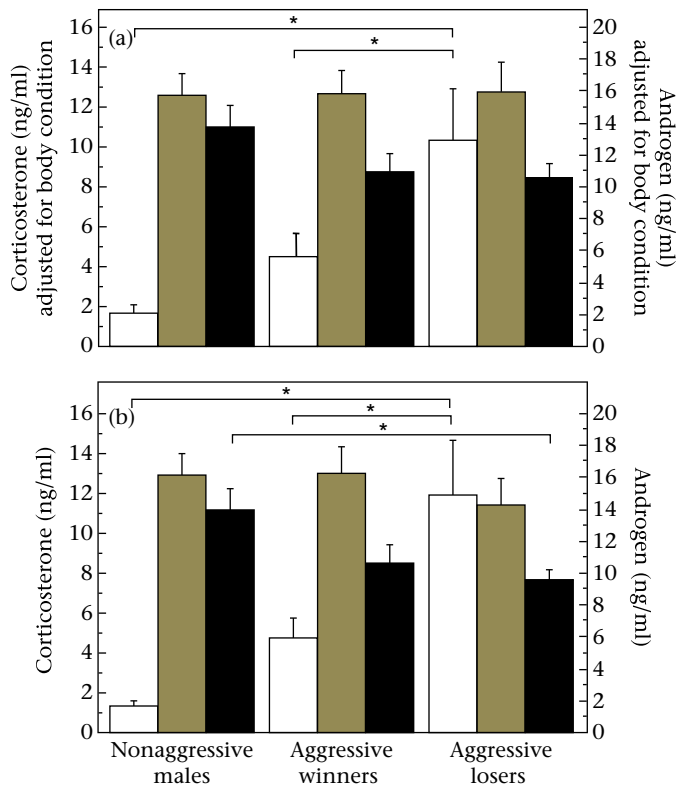


Figure 2. Corticosterone (□), dihydrotestosterone (■) and testosterone (■) levels in nonaggressive males ($N = 31$), aggressive winners ($N = 32$) and aggressive losers ($N = 22$) in natural choruses of *Hyla cinerea*. Hormone levels (a) adjusted for body condition and (b) unadjusted for body condition are shown for comparison (see text). Significant differences are indicated with an asterisk. Whiskers represent standard errors.

(ANOVA: $F_{2,82} = 0.005$, $P = 0.99$; Fig. 2a). Results were similar for DHT values that were not corrected for body condition ($F_{2,82} = 0.51$, $P = 0.60$; Fig. 2b).

There were also no detectable differences between the three groups of males with respect to circulating T levels adjusted for body condition (ANOVA: $F_{2,82} = 2.36$, $P = 0.10$). However, results for T levels that were not adjusted for body condition indicated that there was a difference between groups ($F_{2,82} = 4.27$, $P = 0.01$). In particular, aggressive losers had significantly lower levels of T than nonaggressive males (Tukey–Kramer post hoc test: $P < 0.05$; Fig. 2b), suggesting that body condition contributed to differences in T levels for these two groups.

Effects of Broadcast Close-range Vocalizations on Hormone Levels

There was a significant interaction between treatment and CORT production across the three treatment groups (repeated measures ANOVA: $F_{2,49} = 4.02$, $P = 0.02$; Fig. 3a). Males exposed to broadcast aggressive calls showed a significant increase in circulating CORT levels ($t_{17} = -2.16$, $P = 0.04$). However, pre- and post-broadcast CORT levels did not differ in males exposed to advertisement calls ($t_{17} = -1.03$, $P = 0.3$) or silence ($t_{15} = 1.57$, $P = 0.14$). Despite no detectable changes in CORT levels for males exposed to broadcast advertisement calls and silence, there was a marginally significant interaction for the effects of advertisement calls and silence on CORT production ($F_{1,32} = 3.47$, $P = 0.07$); CORT levels were generally sustained in males exposed to broadcast advertisement calls relative to controls that were exposed to silence (Fig. 3a).

There was a marginally significant interaction between treatment and DHT production (repeated measures ANOVA: $F_{2,49} = 2.77$,

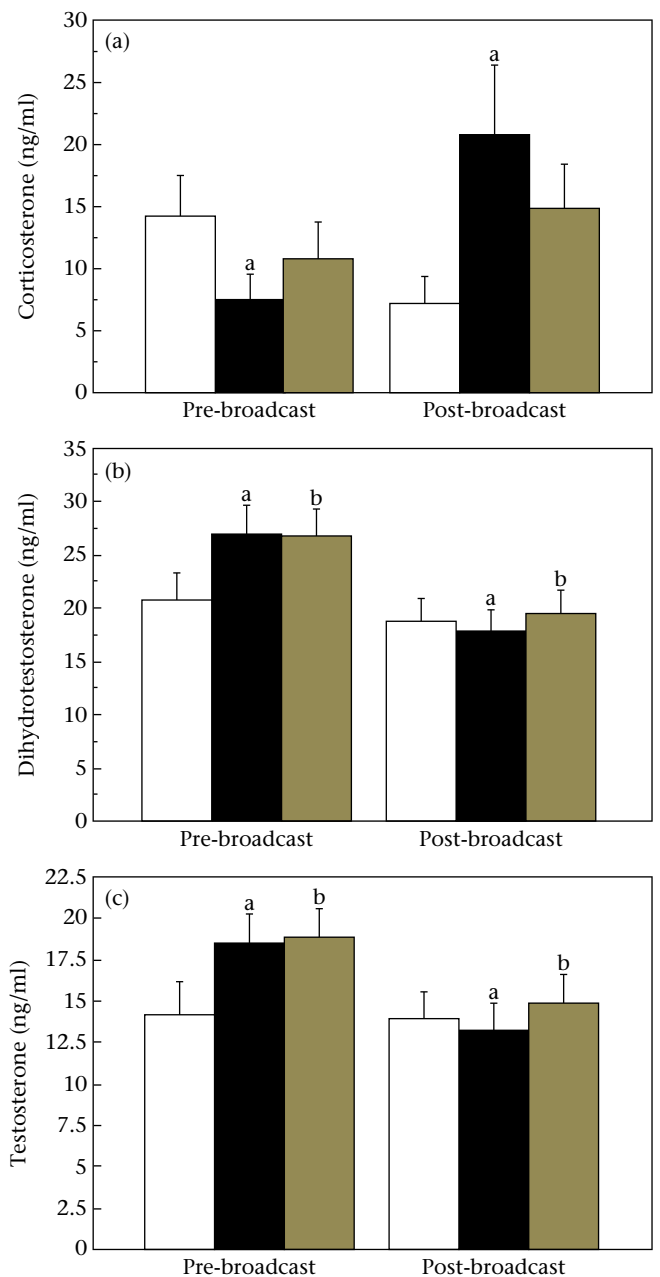


Figure 3. (a) Corticosterone, (b) dihydrotestosterone and (c) testosterone levels in male *Hyla cinerea* prior to (pre-broadcast) and after (post-broadcast) exposure to silence (□, $N = 16$) and to isolated broadcast aggressive calls (■, $N = 18$) and advertisement calls (■, $N = 18$) (duration of stimulus = 45 min, see text for stimulus details). Significant differences in pre- and post-treatment hormone levels within treatment groups are indicated with letters. Whiskers represent standard errors.

$P = 0.07$) and an overall significant difference in pre- and post-broadcast DHT levels across treatment groups ($F_{1,49} = 24.30$, $P < 0.0001$; Fig. 3b). These results were attributable to a significant reduction in DHT in individuals exposed to aggressive calls ($t_{17} = 4.04$, $P = 0.0009$) and advertisement calls ($t_{17} = 2.99$, $P = 0.008$), but not silence ($t_{15} = 1.32$, $P = 0.21$; Fig. 3b).

There was a significant interaction between treatment and T production (repeated measures ANOVA: $F_{2,49} = 3.12$, $P = 0.05$) and an overall significant difference in pre- and post-broadcast T levels across treatment groups ($F_{1,49} = 13.48$, $P = 0.0006$; Fig. 3c). Similar to the results for DHT, these results were attributable to a significant reduction in T for individuals exposed to aggressive calls

($t_{17} = 4.94$, $P = 0.0001$) and advertisement calls ($t_{17} = 2.74$, $P = 0.01$), but not silence ($t_{15} = 0.075$, $P = 0.94$; Fig. 3c).

Size-associated Hormonal Responses to Broadcast Calls

There was no significant relationship between the magnitude of the CORT response to aggressive calls (calculated by subtracting pre-treatment CORT levels from post-treatment CORT levels for each individual) and body size ($F_{1,16} = 0.92$, $r^2 = 0.054$, $P = 0.3$; Fig. 4a). The magnitude of the CORT response to broadcast advertisement calls was, however, significantly and inversely related to male body size ($F_{1,16} = 5.09$, $r^2 = 0.24$, $P = 0.03$), indicating that smaller males showed a greater increase in CORT production in response to these calls (Fig. 4a). Differences in size-related CORT responses to broadcast aggressive calls and advertisement calls were not attributable to differences in the body size of males in the two treatment groups ($t_{34} = -0.265$, $P = 0.79$). The magnitude of the CORT response of control males exposed to silence was not significantly related to body size ($F_{1,14} = 1.14$, $r^2 = 0.07$, $P = 0.3$), indicating that size-related variation in the magnitude of the CORT response of males exposed to broadcast advertisement calls was not attributable to greater CORT production for small males in response to repeated acquisition of blood samples.

The magnitude of the DHT response (calculated by subtracting pre-treatment DHT levels from post-treatment DHT levels for each individual) to aggressive calls was not significantly related to body size ($F_{1,16} = 0.78$, $r^2 = 0.05$, $P = 0.38$). However, the magnitude of the DHT response to advertisement calls was marginally and inversely related to body size ($F_{1,16} = 3.7$, $r^2 = 0.19$, $P = 0.07$), indicating that smaller males (that showed a greater increase in CORT levels in response to broadcast advertisement calls) generally showed a greater reduction in DHT levels than larger males (Fig. 4b). As a result, there was a significant interaction for the effect of call type on the magnitude of the DHT response ($F_{1,32} = 3.86$, $P = 0.05$; Fig. 4b).

The magnitude of the T response to aggressive calls was not significantly related to body size ($F_{1,16} = 0.54$, $r^2 = 0.03$, $P = 0.4$), nor was the magnitude of the T response significantly related to advertisement calls ($F_{1,16} = 1.36$, $r^2 = 0.08$, $P = 0.2$; Fig. 4c). Consistent with these findings, there was no evidence of a significant interaction for the effect of call type on the T response ($F_{1,32} = 1.86$, $P = 0.18$; Fig. 4c).

DISCUSSION

Previous work on green treefrogs showed that chorus sounds (i.e. broadcast recordings of advertisement calls from aggregates of calling males) stimulate sex steroid production in conspecific males (Burmeister & Wilczynski, 2000, 2005). Such effects are likely to impose costs on the signaller by promoting courtship behaviour and/or aggression in competitors. The present study provides evidence that males of this species counter the stimulatory effects of chorus sounds on androgen production in rival males by eliciting elevations in CORT levels during close-range vocal interactions. For example, males in natural choruses that lost aggressive contests had higher CORT levels than aggressive contest winners and nonaggressive males. Elevated CORT levels in aggressive contest losers in natural choruses were associated with low levels of T, but not DHT, suggesting that high CORT levels negatively affected circulating T. However, the difference in T levels for aggressive losers versus nonaggressive males was only detectable when T level was not adjusted for body condition. These results suggest that poor body condition may have contributed to low levels of T in aggressive losers and is consistent with a positive relationship

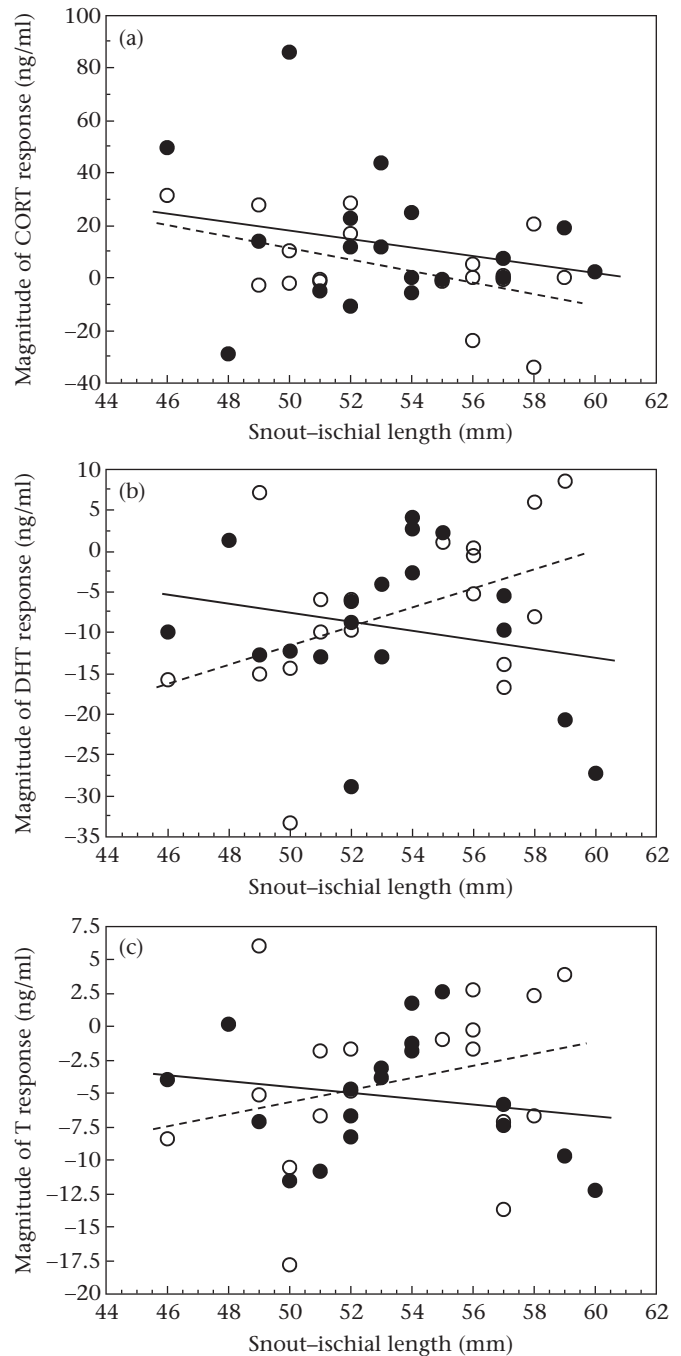


Figure 4. Magnitude of (a) corticosterone, (b) dihydrotestosterone and (c) testosterone response (calculated by subtracting pre-broadcast from post-broadcast hormone levels, shown in Fig. 3) relative to body size (snout–ischial length) in male *Hyla cinerea* exposed to broadcast aggressive calls (solid circles, solid lines) and advertisement calls (open circles, dashed lines).

between body condition and androgen levels reported for this species (Leary & Harris, 2013). In contrast, differences in body condition did not explain differences in CORT levels among groups of males despite the fact that body condition is inversely related to CORT levels in this species (Leary & Harris, 2013) and that aggressive contest losers were generally in the poorest condition. For example, CORT levels were still higher in aggressive losers than in nonaggressive males and aggressive winners even after CORT levels were adjusted for body condition. Overall, results from natural choruses thus indicated that males that lost aggressive interactions

had the highest CORT levels, were the smallest males, were in the poorest condition and had the lowest T levels.

Playback experiments provided a causal link between close-range acoustic signals and differences in circulating hormone levels observed among males in natural choruses. In particular, broadcasts of close-range, isolated aggressive calls resulted in a rapid (45 min) increase in circulating CORT levels in male receivers. A corresponding reduction in both DHT and T levels in males that received broadcasts of aggressive calls was consistent with a negative effect of elevated CORT on circulating androgen levels. The effects of broadcast aggressive signals thus contrasted with those reported for males that were observed to interact aggressively in natural choruses (i.e. androgen levels were not significantly lower in contest losers after correcting for body condition). This discrepancy may be the result of the short period between determination of the outcome of aggressive interactions and when blood samples were obtained (i.e. 5–10 min), a period that may not have been sufficient for a reciprocal interaction between the two steroids to occur. Alternatively, broadcast calls may have had a more robust negative effect on circulating androgen levels because males were exposed to more calls during the playback experiment than when interacting in natural choruses.

In contrast to broadcast aggressive calls, there was no evidence that broadcast isolated advertisement calls or silence affected circulating CORT levels in male receivers. However, the CORT response to advertisement calls was marginally different from the CORT response to silence (i.e. there was evidence for heterogeneity of slopes, wherein circulating CORT levels were sustained in males exposed to broadcast advertisement calls relative to control males exposed to silence; see Fig. 3a). I emphasize this marginally significant result because, when combined with significant size-related differences in the magnitude of the CORT response to broadcast advertisement calls (i.e. the magnitude of the CORT response was greatest for small males; see Fig. 4a), it illustrates how these results could change depending upon the size of signal receivers. This same point can be made for the effects of broadcast advertisement calls on the androgen response of receivers. For example, there was a marginally significant positive relationship between body size of receivers and the magnitude of the DHT response to broadcast advertisement calls (see Fig. 4b), indicating that smaller males generally showed a decrease in DHT levels in response to broadcast advertisement calls, whereas larger males showed an increase in DHT in response to broadcast advertisement calls (a response that is consistent with a greater increase in CORT production in small males in response to advertisement calls). These findings may help explain discrepancies in the results among studies that examine the endocrine responses of male anurans to broadcast advertisement calls (discussed below).

Although CORT levels were only observed to increase in males exposed to broadcast aggressive calls, both broadcast aggressive calls and advertisement calls caused a significant decline in circulating androgen (DHT and T) levels in receivers. One possibility is that circulating CORT levels, which were generally sustained in males exposed to broadcast advertisement calls compared to control males exposed to silence, were high enough or sustained long enough to affect circulating androgens negatively in males exposed to advertisement calls. Alternatively, broadcast advertisement calls may negatively affect circulating androgen levels independently of CORT (see Deviche, Gao, Davies, Sharp, & Dawson, 2012). Regardless, the negative effects of broadcast advertisement calls on circulating androgen levels are intriguing because male advertisement calls typically stimulate androgen production in male anurans (reviewed by: Leary, 2009; Wilczynski & Lynch, 2011), including *H. cinerea* (Burmeister & Wilczynski, 2000, 2005). The results presented here for *H. cinerea* parallel those reported for the Smith

frog, *Hypsiboas faber*. In this species, a reduction in circulating T levels also occurs relatively rapidly in response to broadcast advertisement calls and independently of changes in circulating CORT (de Assis, Nava, Mendonça, & Gomes, 2012).

Such differences in the endocrine responses of receivers to advertisement calls may arise because of differences in the body size of signal receivers (discussed above). Unfortunately, sufficient data are not provided in previous studies to assess this possibility. An alternative explanation for these differences may involve the advertisement call stimuli used in such studies. For example, in all studies reporting a stimulatory effect of advertisement calls on circulating androgen levels in anurans (Burmeister & Wilczynski, 2000, 2005; Chu & Wilczynski, 2001), the advertisement call stimulus consisted of broadcast chorus sounds (consisting of advertisement calls from aggregates of males). In contrast, in the two cases in which advertisement calls negatively affected circulating androgen levels in receivers (de Assis et al., 2012; present study), advertisement calls consisted of isolated calls from individual males that were broadcast at close range. Such contextual differences in the endocrine responses of signal receivers may arise if various call features are effectively masked by the acoustic complexity of natural choruses but unmasked during close-range vocal communication, when the proximity and increased amplitude of the calls effectively isolates them against a backdrop of chorus sounds (i.e. circumstances analogous to the well-known 'cocktail party' problem and 'spatial unmasking'; reviewed by: Bee & Micheyl, 2008; Vélez & Bee, 2011). There are numerous examples, from a wide range of organisms, including many anurans, in which acoustic signals are unmasked when spatially separated (see Bee, 2007, 2008; Bee & Micheyl, 2008; Gerhardt & Klump, 1987, and citations within). Distance between sender and receiver could also alter signal propagation, distortion and attenuation, resulting in the transmission of very different information to signal receivers (reviewed in Bradbury & Vehrencamp, 1998) that could affect the endocrine responses of signal receivers.

The pulsed prefix of the advertisement call (Fig. 1a) is a good candidate feature that could be masked, distorted or attenuated in natural chorus sounds and account for differences in the endocrine responses of male *H. cinerea* to chorus sounds versus and isolated close-range advertisement calls. The pulses of the advertisement call prefix differ from aggressive pulses in duration and repetition rate (Oldham & Gerhardt, 1975) and do not affect the attractiveness of the call (Gerhardt, 1978b), unlike aggressive pulses (Gerhardt, 1978a). By prefacing the advertisement call with a pulsed prefix, male *H. cinerea* could exploit another 'channel' in the auditory pathway that stimulates stress hormone production in signal receivers (i.e. sustains CORT levels) but does not negatively affect attractiveness. Males could benefit by eliciting CORT production in males as well as females if CORT stimulates oviposition (i.e. Radder, Elphick, Warner, & Pike, 2008) and/or decreases female choosiness (i.e. Vitousek & Romero, 2013). These ideas deserve further study.

Agonistic interactions often result in a suite of neuroendocrine responses that affect subsequent behaviour (reviewed by: Adkins-Regan, 2005; Fuxjager et al., 2010; Fuxjager & Marler, 2010; Gleason et al., 2009; Hsu et al., 2006). In *H. cinerea*, CORT administration decreases the propensity to call (possibly via negative effects on circulating androgen levels; Burmeister et al., 2001), and contest losers with the highest CORT levels tend to be the smallest males with the lowest androgen levels that characteristically adopt an alternative noncalling satellite mating tactic (Leary & Harris, 2013). Surprisingly, results from the current study indicate that contest winners and losers do not differ in circulating androgen levels. However, elevated CORT level may be the primary signal mediating suppression of calling behaviour (Leary et al., 2006a, 2006b). High CORT level, for example, can increase the

probability of satellite tactic expression independently of changes in circulating androgens in some anurans (Leary et al., 2006a, 2006b) and may be the key difference between aggressive winners and losers in *H. cinerea*. Size-associated effects of acoustic signals on stress hormone production in receivers (i.e. the magnitude of the CORT response to advertisement calls was greatest for small males) provide a hormonal basis for the typical size discrepancies between calling males and satellite males reported for most anurans (i.e. satellite males are generally smaller; reviewed by Gerhardt & Huber, 2002).

If aggressive calls are more effective at eliciting elevations in circulating CORT levels in signal receivers, why do males characteristically use advertisement calls in natural close-range interactions and only resort to aggressive calls when the interaction escalates? One explanation lies in the fact that males and females are likely to possess sufficiently similar sensory neuroendocrine systems so that signals that effectively repel rival males are likely to negatively affect the attractiveness of the signal to females. Consistent with this hypothesis, aggressive calls, while more effective at eliciting CORT production in male receivers, are also less attractive to females (Gerhardt, 1978a). One solution, rooted in signal optimality theory (reviewed by Bradbury & Vehrencamp, 1998), is to use discrete signals in the context of male–male interactions (but not male–female interactions) that effectively reduce androgen production in rival males. The aggressive calls of *H. cinerea* represent such a signal. For example, males primarily use aggressive signals when the risk of transmission to females is minimal (i.e. during close-range vocal interactions with other males; see Gerhardt, 1978a).

Considerable emphasis has recently been placed on the dichotomy of hormonal outcomes associated with social interactions: one in which androgen production is stimulated (i.e. consistent with the challenge hypothesis; Wingfield et al., 1987, 1990, 1999) and one in which androgen production is suppressed via elevated glucocorticoids (reviewed by Soto-Gamboa, Villalón, & Bozinovic, 2005). The latter effects appear to be associated predominately with social dominance and instability (reviewed in: Creel et al., 2013; Hsu et al., 2006; Soto-Gamboa et al., 2005). Several investigators have also reported no effect of social challenges on circulating androgens (Apfelbeck & Goyman, 2011; Deviche et al., 2014; Deviche, Sharp, et al., 2012; Fokidis, Orchinik, & Deviche, 2011). Few studies, however, have examined contextual differences in the endocrine responses of receivers to signals used in both courtship and aggressive interactions (but see Fokidis et al., 2011, for an example involving endocrine responses of birds in urban versus natural habitats). It would be interesting to know whether cases that are inconsistent with predictions of the challenge hypothesis are associated with contextual differences in transmission of the signals (i.e. close-range versus long-range communication) or ‘hidden’ features that are unmasked during close-range communication and that minimize the production of androgens in male receivers (i.e. see Arnqvist, 2006). Such studies are likely to provide insights into the evolution of sexual signals and how males potentially circumvent the conflict associated with stimulatory effects of courtship signals on androgen production in male receivers.

The effects of close-range vocal signals on the endocrine physiology of signal receivers reported here for *H. cinerea* may be particularly important in understanding models to explain the endocrine basis for calling behaviour in anuran amphibians. For example, the energetics hormone–vocalization (EHV) model (Emerson, 2001), which was based on concepts of the challenge hypothesis (Wingfield et al., 1990), proposes that interactions among chorusing conspecific males result in a temporal increase in circulating androgen levels that mediates an increase in vocal effort

(i.e. the energy invested in calling behaviour). An increase in vocal effort should, in turn, increase the rate of energy depletion that stimulates the production of CORT. CORT is expected to eventually reach threshold levels (i.e. when energy reserves are depleted) that negatively affect circulating androgen levels and suppress vocal behaviour. While steroid hormone profiles for *H. cinerea* are consistent with predictions of the EHV model (i.e. noncalling ‘satellite’ males have higher CORT levels and lower androgen levels than calling males; see Leary & Harris, 2013), the results presented here suggest that temporal changes in hormone levels and the interrelationships among circulating hormone levels and energetic state may be inconsistent with model predictions. For example, circulating androgen levels may not increase over sequential nights of calling activity if aggressive interactions suppress androgen production (see Leary, Garcia, & Knapp, 2008; Leary, Garcia, Knapp, & Hawkins, 2008). Moreover, the effects of aggressive interactions on circulating CORT levels could effectively decouple the predicted relationship between CORT level and energy reserves. Additional studies are needed to assess whether aggressive interactions result in inconsistencies with predictions of the EHV model.

Conclusions

In summary, male green treefrogs appear to elicit an increase in circulating CORT levels in rival males during close-range vocal exchanges that counter the stimulatory effects of chorus sounds on androgen production. Males benefit from eliciting a stress response in male receivers because rival males are effectively ousted from the pool of conspecifics engaged in vocal behaviour (i.e. elevated CORT levels suppress calling behaviour). The counter strategy for males, particularly small individuals, is to adopt a noncalling satellite mating tactic.

While there are certainly circumstances in which stimulation of androgen production in rival males could benefit signallers (e.g. if increased androgens decrease parental care; Ketterson, Nolan, Wolf, & Zeigenfus, 1992; Lynn, 2008; Wingfield et al., 1990), such circumstances are not applicable to *H. cinerea* (which shows no parental care). Along these same lines, signallers could gain advantages by stimulating androgen production in receivers if elevated androgens suppress immune responses (Folstad & Karter, 1992). Such effects, however, are controversial (Hillgarth & Wingfield, 1997; Roberts, Buchanan, & Evans, 2004) and seem unlikely to outweigh the costs associated with androgenic effects on courtship behaviour and aggression in rival males. Signallers thus probably have little to gain by stimulating androgen production in rival males and are expected to evolve strategies that effectively suppress androgen production in male receivers. The present study suggests that calls produced by male green treefrogs during close-range vocal interactions can effectively increase CORT levels and suppress androgen production in rival males.

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