

**THE EFFECTS OF ACOUSTIC SIGNALS ON THE
ENDOCRINE PHYSIOLOGY OF FEMALE GREEN
TREEFROGS, *HYLA CINEREA***

by
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Abstract

The vocalizations of male *H. cinerea* stimulate cortisol production in male receivers during close-range interactions. In many vertebrate species, males and females possess similar sensory neuroendocrine pathways, suggesting that acoustic signals may also stimulate CORT production in females. Males of this species would potentially benefit from stimulating CORT production in females because elevated CORT levels significantly decrease the strength of female preferences for energetically costly calls (i.e., calls produced at rapid rates). The primary objective of my honors research was to assess how the acoustic signals produced by male green treefrogs, *Hyla cinerea*, influence the endocrine physiology of female signal receivers. Specifically, I assessed how male acoustic signals alter circulating levels of stress hormones (i.e., glucocorticoids) in females. My results were unexpected in that advertisement calls actually decreased circulating levels of CORT in female receivers, indicating that males do not manipulate the stress physiology of females in ways that would allow them to gain access to females. My research thus suggests that there are neuroendocrinological differences between males and females that result in very different endocrine responses to the same acoustic signals. Another interesting outcome that emerged from my data involved the effect of aggressive calls on the endocrine physiology of females. Results indicated that exposure to aggressive calls resulted in decreased levels of progesterone and a marginally significant reduction in estradiol. These results may explain why males of this species only use aggressive signals in the context of male-male interactions;

female reception of aggressive calls would decrease hormones associated with female sexual behavior, and thus decreases the likelihood of reproductive success for calling males.

LIST OF ABBREVIATIONS

Corticosterone- CORT
Progesterone- P
Testosterone- T
Dihydrotestosterone- DHT
Hypothalamic-pituitary-adrenal axis- HPA axis

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INTRODUCTION

The effects of male courtship signals on the endocrine physiology and behavior of females have traditionally been viewed from a cooperative standpoint. For example, male courtship signals often stimulate estradiol production that promotes receptivity in females and synchronizes reproductive activity between the sexes (Chu and Wilczynski, 2001; Lea et al., 2001; Wilczynski and Lynch, 2001; Burmeister and Wilczynski, 2006; Adkins-Regan, 1998, 2005; Lynch and Wilczynski, 2006; Wingfield and Marler, 1988; Maney et al., 2007). Male sexual signals probably vary in the extent to which they stimulate sex steroid production, thereby providing a hormonal basis for female preferences that can drive the evolution of male sexual signals (Andersson, 1994; Leary 2015).

The primary objective of my honors research was to assess how the acoustic signals produced by male green treefrogs, *Hyla cinerea*, influence the endocrine physiology of female signal receivers. Specifically, I assessed how male acoustic signals alter circulating levels of stress hormones (i.e., glucocorticoids) in females. The rationale for pursuing this question stemmed from previous endocrine-based work in this species. For example, previous work showed that the acoustic signals produced by males of this species stimulate the production of glucocorticoids in conspecific males (Leary, 2014). Males benefit from eliciting glucocorticoid production in other males because high glucocorticoid levels suppress androgen production that decreases the probability of vocalization in rival males (Leary, 2014). Males thus increase their chances of acquiring mates by eliciting glucocorticoid production in other males because high glucocorticoid levels effectively oust competing males from the pool of conspecifics engaged in vocalization (Leary, 2014). Males and females of most vertebrate species possess highly

conserved sensory neuroendocrine pathways (Adkins-Regan, 2005) suggesting that the acoustic signals produced by males of this species may also stimulate glucocorticoid production in females. But what would male green treefrogs potentially gain by eliciting glucocorticoid production in females? Recent work has shown that elevated glucocorticoid levels can have a surprising effect on female mate choice behavior in this species. For example, high corticosterone (CORT, the primary glucocorticoid in amphibians) levels abolish female preferences for calls produced at high rates, which are energetically costly for males to produce and are preferred by females with low CORT levels (Davis and Leary, 2015). Unattractive males could thus gain access to mates by producing acoustic courtship signals that stimulate CORT production in females. If so, such results would provide new perspectives on how male sexual signals alter the endocrine physiology of females that would shift the traditional cooperative paradigm (involving the production sex steroids) to one that includes manipulation of females by males (involving the production of stress hormones). The neuroendocrine effects of male sexual signals on female receivers could thus provide new insight into the evolution of such signals.

Acoustic Communication in Anurans

The primary mode of communication for anurans (frogs and toads) involves the production of acoustic signals. For example, males of many anuran species produce advertisement calls to attract females, and both advertisement and aggressive vocalizations to deter rival males (Sullivan, 1983; Gerhardt and Huber, 2002). Male green treefrogs (*H. cinerea*) produce both advertisement and aggressive calls (Fig. 1). These two calls are distinct in that the advertisement call is mostly unpulsed (i.e., not amplitude modulated), except for a brief prefix at

the onset of the call, whereas the aggressive call is highly amplitude-modulated throughout the duration of the call (Oldham and Gerhardt, 1975; Gerhardt, 1978; see Fig. 1).

Production of advertisement and aggressive calls varies with social context (Gerhardt, 1978; Oldham and Gerhardt, 1975)(Fig. 1). For example, detection of a male in another male's territory typically elicits the production of advertisement calls that are directed at the intruding male. If the encroaching individual doesn't retreat, the interaction may escalate to include the production of aggressive calls (*reviewed in* Gerhardt and Huber, 2002; see also Reichert, 2011; Reichert and Gerhardt, 2013). Although aggressive calls are typically produced in the context of male-male interactions, female *H. cinerea* will typically evaluate males for a long period of time (i.e. several hours) before choosing a mate. During this time, they are often exposed to both advertisement calls and aggressive calls associated with male-male interactions (*reviewed in* Gerhardt & Huber, 2002).

Endocrine Effects of Acoustic Signals in Anurans

The advertisement calls of male anurans are known to increase receptivity in female receivers by increasing the activity of hypothalamic neurons (Allison, 1992; Wilczynski and Allison, 1989) and stimulating estradiol production (Lea et al., 2001; Lynch and Wilczynski, 2005; Wilczynski and Lynch, 2011). Advertisement calls can have similar stimulatory effects on the endocrine system of males as well. For instance, chorus sounds (i.e., sounds of aggregates of advertising males) are known to activate gonadotropin-releasing hormone neurons (GnRH) that stimulate the hypothalamic-pituitary-gonadal (HPG) axis and increase the production of circulating androgens in males (Burmeister and Wilczynski, 2000, 2001, 2005).

Because advertisement calls stimulate androgen production in other males, the production of such calls can be disadvantageous for the signal sender in that it increases aggression and sexual behavior in rival males, and thus, increases competition for mates (Leary, 2014). However, advertisement calls may only stimulate androgen production in males when they are heard in the context of a chorus. For example, when advertisement calls are isolated and broadcast to simulate close-range vocal interactions, both advertisement and aggressive calls of male *H. cinerea* negatively affect circulating androgen levels (Leary, 2014). The difference in endocrine responses of signal receivers to chorus sounds (consisting of advertisement calls from aggregates of calling males) versus isolated close-range advertisement calls may be related to the ‘cocktail party’ problem wherein various features of acoustic signals are effectively masked by the complexity of the acoustic environment (Leary, 2014). Studies of many different species including anurans, for example, have found that various features of acoustic signals can be effectively unmasked when spatially separated (*reviewed by* Bee and Micheyl, 2008; Velez and Bee, 2011). Spatial unmasking of acoustic features during close-range vocal communication could account for differences in the endocrine response of male *H. cinerea* to chorus sounds versus isolated advertisement calls (Leary, 2014). In particular, the pulsed prefix of the advertisement call may be a key feature that suppresses androgen production in male receivers and is unmasked during close-range vocal interactions (Leary, 2014).

In addition to changes in sex steroid levels in signal receivers, male calls can also influence circulating levels of stress hormones. In *H. cinerea*, for example, close-range isolated advertisement and aggressive calls stimulate CORT production in male receivers. Aggressive calls are generally much more effective at stimulating CORT production in males than advertisement calls, but advertisement calls can have similar effects, particularly in small males

(Leary, 2014). Elevated CORT typically negatively affects the production of sex steroids by inhibiting enzymes involved in the synthesis of androgens and/or by stimulation of gonadotropin inhibitory hormone (Calisi et al., 2008; Chand and Lovejoy, 2011; Kirby et al., 2009), suggesting that a similar reciprocal interaction between the two steroids could account for the reduction in sex steroids in male receivers exposed to isolated broadcast calls (Leary, 2014). The combination of high levels of CORT and low levels of androgens is associated with a reduction in the probability that the male will vocalize (Leary et al., 2006). Therefore, males may benefit from stimulating CORT production in competing males because it effectively removes these males from the pool of conspecifics engaged in chorus behavior (Leary, 2014).

Because there is a significantly greater stress response to aggressive calls than to advertisement calls in males (Leary, 2014), amplitude modulations (i.e., pulses) may be particularly important in stimulating stress hormone production in signal receivers. Pulses are incorporated into the prefix of the advertisement call of *H. cinerea* (see Fig. 1). This feature of the advertisement call may be unmasked during close-range vocal communication and could stimulate stress hormone production in both males and females. Importantly, however, reproductive females of most vertebrate species generally do not exhibit a reciprocal interaction between glucocorticoids and circulating sex steroids (Legato and Bilezikian, 2004) suggesting that stimulation of glucocorticoid production should not negatively influence circulating sex steroid levels (and thus receptivity in females). My honors thesis work examined whether the vocal signals of *H. cinerea* stimulate CORT production in females, as they do in males.

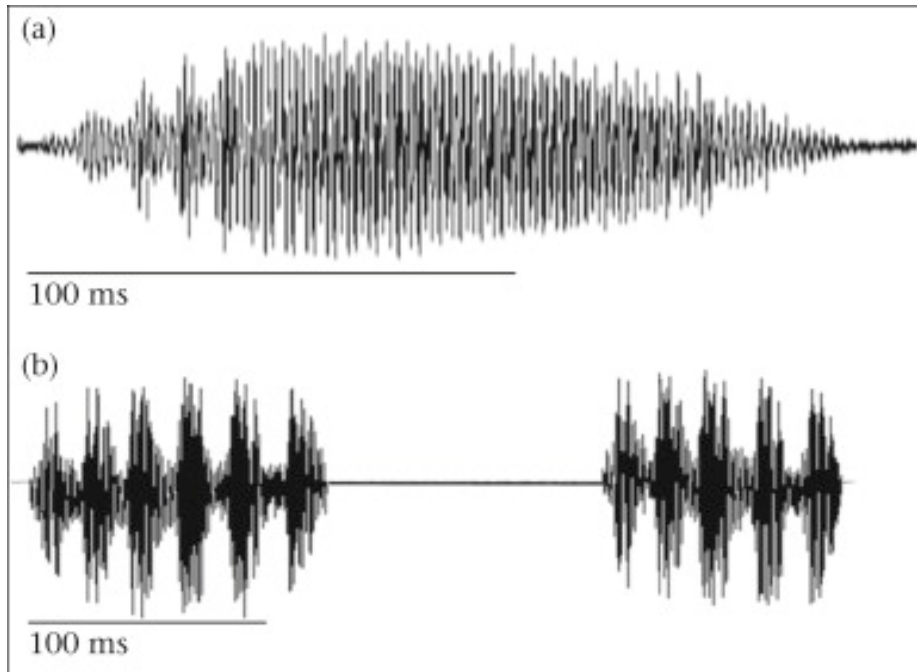


Figure 1: Waveform representations of (a) advertisement and (b) aggressive calls from *Hyla cinerea* (from Leary, 2014).

Female Mate Preferences in Hyla cinerea

There are two primary factors that can affect mate choice by females: ‘preference functions’ (how attractive a mate is ranked) and choosiness (the effort made to distinguish the best mate) (Jennions and Petrie, 1997). In species where there is little paternal involvement following copulation, as in most anuran amphibians, females presumably select males with traits that increase offspring fitness (Sherman et al., 2010). Females potentially assess mate quality by discriminating between/among male acoustic signals. Female *H. cinerea* typically show a strong preference for advertisement calls that are produced at high rates, calls that are longer in duration, and calls that are louder (i.e., signals are more energetically taxing for the male, Gerhardt, 1987; Murphy and Gerhardt 1996; Taigen et al., 1985; Prestwich, 1994). By choosing

males that produce calls that are energetically demanding, females potentially gain indirect benefits that increase offspring viability (Welch et al., 1998).

Although female preferences are consistent most of the time, a female cannot always afford to be so selective. Environmental stressors such as inadequate food availability or increased risk of predation are often linked to a decrease in female choosiness (Jennions and Petrie, 1997). Such stressors typically stimulate the neuroendocrine stress axis, which increases circulating levels of CORT (Hennessy and Levine, 1978). Recent studies have indicated that circulating stress hormone levels can mediate a reduction in female preferences. For example, female *H. cinerea* that received CORT injections showed a significant decrease in the strength of preferences for male acoustic signals (Davis and Leary, 2015). Therefore, unattractive males could increase their chances of acquiring a mate by stimulating CORT production in females. My study explored this hypothesis by examining how the acoustic signals produced by male green treefrogs affect the endocrine physiology of females. I predicted that advertisement and aggressive calls produced by males would increase circulating levels of glucocorticoids in female *H. cinerea*.

METHODS AND MATERIALS

I captured female *H. cinerea* during the 2014 breeding season from late May to early August at the University of Mississippi Field Station (Lafayette County, MS, USA). Females were then transported in cloth bags to a lab located on the University of Mississippi campus within one hour. Blood samples (75-100 μ l) were then obtained via cardiac puncture (< 5 min) using a 27-gauge heparinized hypodermic needle.

After the initial blood draw, females were immediately placed in separate 38-litre glass aquaria in a dark room. Females were then exposed to one of the following treatments for 45 min: 1) broadcast isolated advertisement calls, 2) broadcast isolated aggressive calls, 3) broadcast random tones, or 4) silence. The random tones treatment served as an additional control to assess whether potential hormonal effects of acoustic signals were related to the perception of sound in general or were specific to the calls produced by this species. I broadcast the acoustic signals using a SME-AFS Amplified Field Speaker (Saul Mineroff Electronics, Inc, Elmont, NY, USA) at the amplitude of 90 db SPL, reflecting the approximate amplitude of natural calls for *H. cinerea* (Humfeld, 2013; Leary, 2014).

The advertisement call stimulus was comprised of a single isolated advertisement call recorded from a male that produced peak carrier frequencies of 800 and 2600 Hz, which approximated the mean values for the study population (mean low peak carrier frequency = 788 Hz, range 640-1030; mean high peak carrier frequency = 2550 Hz, range 1724-3600 Hz, see Leary, 2014). Call duration (189 ms) and intercall interval (612 ms) of the advertisement stimulus was well within the range of natural calls for this species as well (mean call duration = 145 ms, range 86- 221 ms; mean intercall interval = 456, range 255-800 ms, see Leary, 2014).

The aggressive call stimulus was created using two consecutive aggressive calls from an individual that produced similar peak frequencies as the advertisement call (within 60 Hz) with durations of 120 ms and 100 ms. Aggressive calls were digitally manipulated using Raven software (Cornell Bioacoustics Laboratory, Ithaca, NY, U. S. A) (see Leary, 2014) so that the calls played in rapid succession, creating a series of four consecutive calls separated by 120ms. Each series of four consecutive calls was separated by 1705 ms and were broadcast at the same amplitude as the advertisement call (i.e. 90dB SPL).

Lastly, I used “random” tones that fell outside the range of frequency of the acoustic signals produced by this species (i.e., consisting of 400, 4000, 2100, 1200, 300, 1800, and 4200 Hz) with tone durations of 120 ms and intervals between successive tones that equaled the interval between successive advertisement calls (i.e., 612 ms). Hence, the total energy for each acoustic stimulus was similar across treatments.

After females were exposed to one of the acoustic stimuli (or silence) for 45 minutes, they were quickly (within 5 minutes) bled a second time and body size (snout-ischial length, SIL) and weight were then measured. Samples were then centrifuged for 5 min at 3,000 rpm. I exposed 18 females to advertisement calls, 4 to aggressive calls, 8 to random tones, and 7 to silence. Sample sizes are small in some instances due to difficulty in finding sufficient numbers of females in the field and/or difficulty in extracting sufficient blood samples from small experimental subjects within the appropriate time frame of 5 min (e.g., mean weight of females =11.1g, mean SIL=55mm). After the experiment was completed, all subjects were released at the site of capture. Plasma samples were stored at -20C until they were used to quantify hormone levels using radioimmunoassay (see below).

Permits to conduct this research were obtained through the Mississippi Department of Wildlife, Fisheries and Parks and approved by the UM Animal Care and Use Committee.

Column Chromatography and Radioimmunoassay Procedures

Plasma hormones were separated and quantified using the procedure described in Leary and Davis (2013). Plasma samples were incubated overnight with tritiated hormone (PerkinElmer, Inc. Hebron, KY, USA) 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 2%, 40%, and 50% ethyl acetate in iso-octane were then used to collect progesterone (P), estradiol (E2) and CORT, respectively. Fractions were dried under nitrogen and resuspended in phosphate-buffered saline containing 0.3% gelatin for radioimmunoassay. Antibodies were purchased from the following suppliers: P, Fitzgerald Industries International, Inc (Acton, MA, USA); CORT, MP Biomedicals, LLC (Solon, OH, USA); E2, ABD Serotec (Raleigh, NC, USA). Plasma samples were analyzed for hormone levels in two assays.

Statistics

A total 74 plasma samples from 37 female *H. cinerea* were examined to determine the effects of acoustic signals on their endocrine physiology. I compared the effects of different treatment groups (i.e. advertisement calls, aggressive calls, random tones, and silence) on pre- and post-treatment hormone levels (CORT, E2, and P) using paired t tests.

Size-associated variation in the magnitude of the CORT response to advertisement calls has previously been reported for males of this species (i.e., small males show a greater stress

response than large males, Leary 2014). Therefore, I examined the relationship between the magnitude of the CORT response to advertisement calls and body size in females using a linear regression analysis. The magnitude of the CORT response was calculated by subtracting pre-treatment CORT levels from post-treatment CORT levels for each individual.

RESULTS

Effects of Acoustic Signals on the Endocrine Physiology of Females

CORT levels

Females exposed to advertisement calls showed a significant decrease in circulating levels of CORT ($t_{17} = 2.04$, $p = 0.05$; Fig. 2). None of the other stimuli had any effect on circulating CORT level (aggressive call $t_3 = 0.31$, $p = .78$; random tones $t_7 = 0.03$, $p = 0.98$; silence $t_6 = -0.64$, $p = 0.55$) (see Fig. 2).

Estradiol levels

Females exposed to aggressive calls showed a marginally significant decrease in E2 ($t_3=2.91$, $p=0.06$; Fig. 2b). None of the other stimuli had any effect on circulating E2 levels (advertisement call $t_{17}=1.82$, $p=0.10$; random tones $t_7=1.03$, $p=0.34$; silence $t_6=1.65$, $p=0.15$).

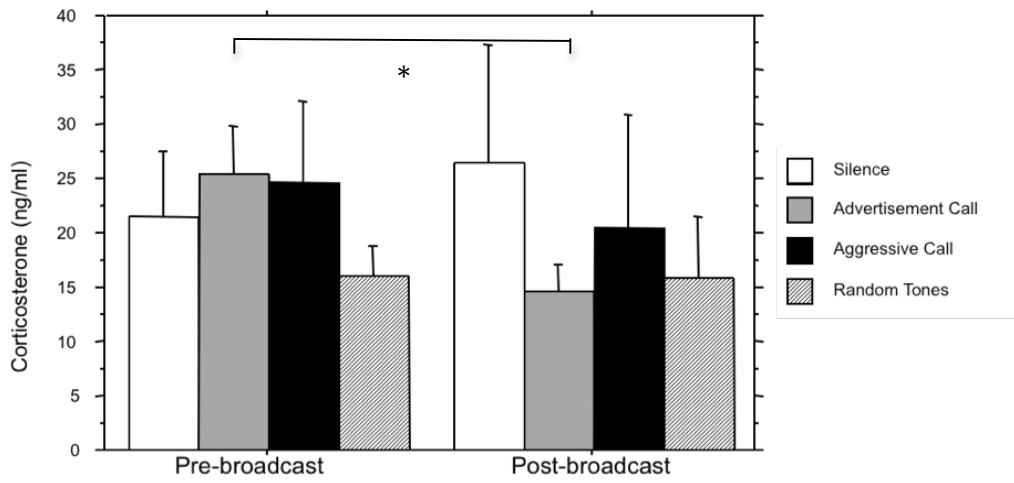
Progesterone levels

Females exposed to aggressive calls showed a significant decrease in P ($t_3=7.41$, $p=0.01$; Fig. 2c). None of the other stimuli had any effect on circulating P level (advertisement call $t_{17}=0.85$, $p=0.41$; random tones $t_7=0.48$, $p=0.65$; silence $t_6=1.87$, $p=0.11$).

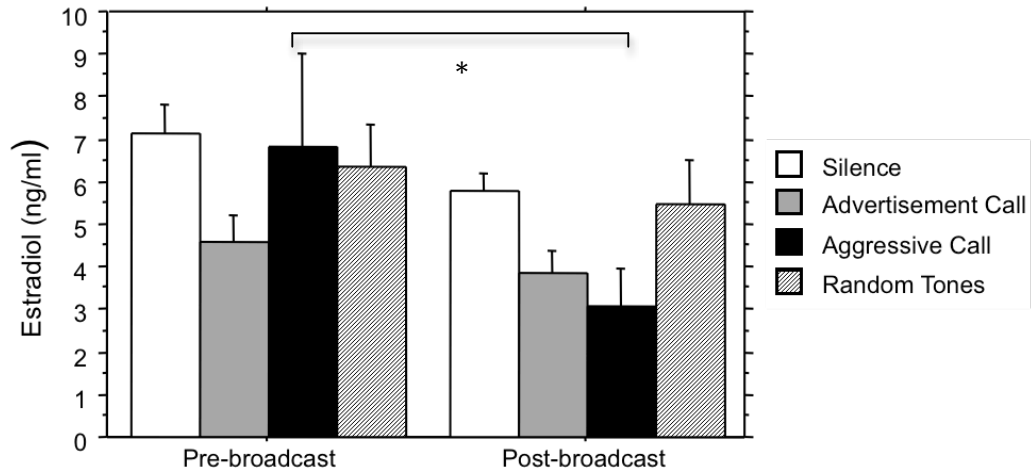
Size-associated Hormonal Responses to Advertisement Calls

Linear regression analysis indicated no significant relationship between the magnitude of the CORT response and body size (SIL) in females exposed to broadcast advertisement calls ($F_{1,16} = 0.90$, $r^2 = 0.053$, $p = 0.36$; Fig. 3).

A.



B.



C.

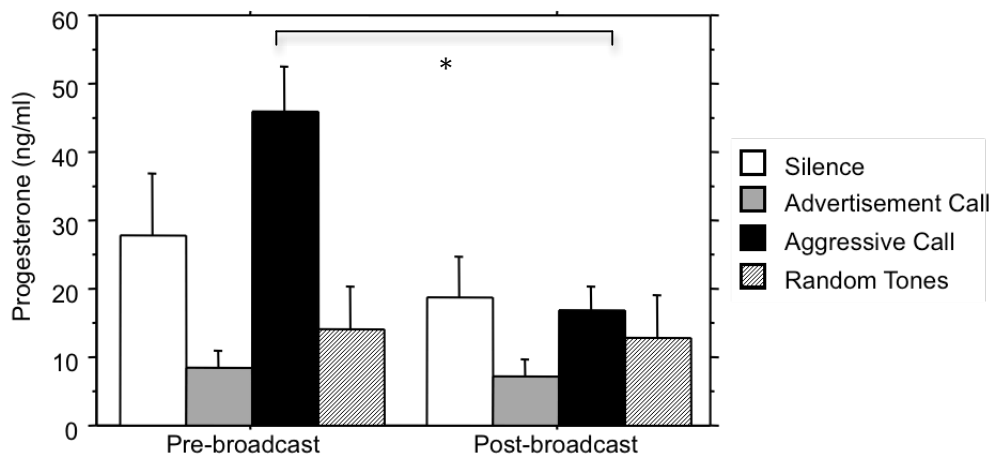


Figure 2: (a) Corticosterone, (b) estradiol, and (c) progesterone) levels before and after exposure to silence (n=7), advertisement calls (n=18), aggressive calls (n=4), and random rones (n=8) in female *Hyla cinerea*. Significant differences are indicated with asterisk. Whiskers indicate standard errors.

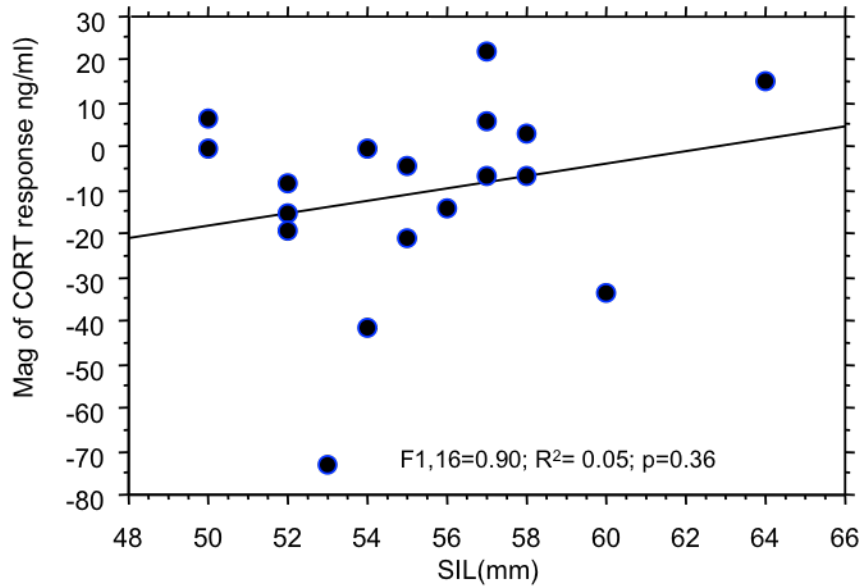


Figure 3: Regression analysis examining the magnitude of the CORT response in relation to body size (SIL in mm) in female *H. cinerea*.

DISCUSSION

Previous research on green treefrogs indicated that the vocalizations of male *H. cinerea* stimulate CORT production in male receivers during close-range interactions (Leary, 2014). In many vertebrate species, males and females possess similar sensory neuroendocrine pathways (Adkins-Regan, 2005) suggesting that acoustic signals may also stimulate CORT production in females. Males of this species would potentially benefit from stimulating CORT production in females because elevated CORT levels significantly decrease the strength of female preferences for energetically costly calls (i.e., calls produced at rapid rates, Davis and Leary, 2014). This previous work provided the basis of my research.

My results were unexpected in that advertisement calls actually decreased circulating levels of CORT in female receivers, indicating that males do not manipulate the stress physiology of females in ways that would allow them to gain access to females. Moreover, in contrast with the inverse relationship between the magnitude of CORT response and body size found in males exposed to advertisement calls (Leary, 2014), regression analysis revealed no significant relationship between the magnitude of the CORT response and body size in females. My results thus suggest that there are neuroendocrine differences between males and females that result in very different endocrine responses to the same acoustic signals. The contrasting hormonal responses between males and females suggest that female's sensory pathways may have evolved differently so that male courtship signals do not evoke a stress response, as they do in rival males. Because a stress response decreases a female's preference for attractive males (Leary and Davis, 2014), resistance to hormonal manipulation may be beneficial to the female and the fitness of her offspring. Females may have evolved to interpret male advertisement calls in a way that maximizes their discriminatory abilities by decreasing their levels of CORT.

Another interesting outcome that emerged from my data involved the effect of aggressive calls on the endocrine physiology of females. Results indicated that exposure to aggressive calls resulted in decreased levels of progesterone and a marginally significant reduction in estradiol, which may explain the limited context in which males use aggressive calls. A reduction in progesterone and estradiol has been shown to negatively affect the probability that a female will be sexually responsive to males (Lynch et al., 2006). This reduction in sexual behavior is costly for males because it decreases the probability of attaining a mate. Thus, it would be disadvantageous to use aggressive calls in close proximity to potential mates. Males seem to avoid this problem by only using aggressive calls during male-male close range interactions (*reviewed in* Gerhardt and Huber, 2002; see also Reichert, 2011; Reichert and Gerhardt, 2013).

Although my data provide some evidence that aggressive signals can have a dramatic effect on the endocrine physiology of females that are likely to contribute to the signaling strategies of males, additional work that includes larger sample sizes is required before any conclusions can be drawn. For example, there was considerable variation in circulating hormone levels among individuals prior to exposure to broadcast signals, particularly with respect to progesterone levels. For unknown reasons, pretreatment progesterone levels were very high in the group of females exposed to aggressive signals, thereby increasing the likelihood of a detectible decrease in the same hormone.

In conclusion, although research has been conducted on the hormonal response to acoustic communication in male *H.cinerea*, there has been little research on how the reception of acoustic signals affects females, and particularly their CORT response. My results link together previous studies examining the effect of acoustic signals on hormone production and show that there are differences in the endocrine responses of males and females. For example, unlike

males, females show a significant reduction in circulating CORT levels in response to hearing male advertisement calls. These results suggest that males of this species do not manipulate the stress physiology of females to gain access to them. I also found some evidence that aggressive calls can significantly reduce circulating sex steroids in females. Such effects are likely to reduce receptivity in females and decrease mating success in males. These effects are likely to contribute to the fact that males rarely produce aggressive signals in the presence of females.

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