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EXAMINING THE POTENTIAL ROLE OF REPRODUCTIVE INTERFERENCE IN THE  
DECLINE OF NATIVE GREEN TREEFROGS FOLLOWING CUBAN TREEFROG  
INVASIONS

A Thesis  
Presented in partial fulfillment of requirements  
for the degree of Master of Science  
in the Department of Biology  
The University of Mississippi

by

JOSEPH G.C. KENNEDY

December 2019

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## ABSTRACT

Reproductive interference occurs when the mating behaviors of one species negatively impact the fitness of another species. It is of increasing interest in invasive species biology because the introduction of alien species often leads to novel sexual interactions with native taxa, which can contribute to their decline. I examined whether reproductive interference plays a role in the decline of native green treefrogs (*Hyla cinerea*) following invasions of Cuban treefrogs (*Osteopilus septentrionalis*) in the southeastern United States. The impetus for this study revolves around similarities in spectro-temporal features of Cuban treefrog courtship calls and green treefrog aggressive calls. The significance of this similarity is that the aggressive calls of green treefrogs stimulate elevations in circulating glucocorticoids in rival conspecific males, which suppresses androgen production and reproductive behavior. I thus hypothesized that introduced Cuban treefrogs negatively impact green treefrogs because the courtship calls of Cuban treefrogs stimulate chronic elevations in circulating glucocorticoid levels that suppress reproduction in native green treefrogs. This hypothesis was tested using vocal playback experiments to examine the effects of Cuban treefrog calls on the endocrine physiology of green treefrogs and by examining hormone levels and calling behavior of green treefrogs in natural choruses with and without Cuban treefrogs. Playback experiments revealed that the aggressive calls of green treefrogs stimulate glucocorticoid production in conspecific males, consistent with previous work, but that Cuban treefrog vocalizations do not stimulate glucocorticoid production in green treefrogs. In natural choruses, the density of calling male green treefrogs and the proportion of non-calling male green treefrogs were positively correlated with circulating glucocorticoids and negatively

correlated with androgens, and body size was positively correlated with circulating androgen levels. After statistically controlling for these variables, there was no evidence that circulating hormone levels or behavior differed in choruses of green treefrogs in the presence and absence of Cuban treefrogs. These results suggest that interactions among competing conspecific male green treefrogs influence circulating hormone levels but that Cuban treefrogs are not altering the endocrine physiology of green treefrogs.

## DEDICATION

I would like to dedicate this thesis to my friends, family, and colleagues whose encouragement made it possible for me to complete this research.

## LIST OF ABBREVIATIONS

SIL	Snout-ischial length
DHT	Dihydrotestosterone
T	Testosterone
CORT	Corticosterone
ANOVA	Analysis of variance

## ACKNOWLEDGEMENTS

The research presented in this thesis was supported by grants through the Chicago Herpetological Society, the Society for the Study of Amphibians and Reptiles, and the University of Mississippi Field Station. I thank James Perran Ross, Laura Gaudette, Jiri Hulcr, and Andrea Lucky for allowing me to sample green treefrogs on their property. I would like to thank my advisor, Christopher Leary, for mentoring me and helping me become a better writer and researcher. In addition, he assisted me with field research, laboratory work, and provided a lot of moral support. I would also like to thank my committee, William Resetarits and Stephen Brewer for their advice and contributions to my thesis. In addition, I would like to thank Steven Johnson at the University of Florida and Jason Rohr at the University of South Florida for assisting me with locating sampling sites and providing advice. I would especially like to thank Leigha Coke, Anthony Messina, Sarah Crocker-Buta, Sean Manz, and Max Maddox for assistance with field work. Without them, field work would have been much more challenging and far less enjoyable. I would like to extend a special thanks to Stephen Brewer and Sarah Crocker-Buta for assisting me with the statistics for my research. Lastly, I would like to thank my friends and family, particularly Elizabeth Thomas and Matthew Wronsky, along with my mother and father for cheering me on in my times of struggle and success. I could not have accomplished this without them.



## TABLE OF CONTENTS

ABSTRACT	ii
DEDICATION	iv
LIST OF ABBREVIATIONS	v
ACKNOWLEDGEMENTS	vi
LIST OF FIGURES	ix
I. EXAMINING THE POTENTIAL ROLE OF REPRODUCTIVE INTERFERENCE IN THE DECLINE OF NATIVE GREEN TREEFROGS FOLLOWING CUBAN TREEFROG INVASIONS	
Introduction	1
II. CHAPTER ONE: INVESTIGATING THE EFFECT OF BROADCAST CUBAN TREEFROG CALLS ON HORMONE LEVELS IN NATIVE GREEN TREEFROGS	
Methods	7
Results	13
Discussion	15
III. CHAPTER TWO: HORMONE LEVELS AND BEHAVIOR OF GREEN TREEFROGS IN NATURAL CHORUSES WITH AND WITHOUT CUBAN TREEFROGS	
Methods	18
Results	21

Discussion	27
Overall Conclusions	28
<b>BIBLIOGRAPHY</b>	<b>30</b>
<b>VITA</b>	<b>36</b>

## LIST OF FIGURES

Figure 1. Representative advertisement call of Cuban treefrog and aggressive call of green treefrog	5
Figure 2. Acoustic stimuli used in acoustic playback experiment	9
Figure 3. Corticosterone levels prior to and following exposure to broadcast stimuli	14
Figure 4. Total androgen levels prior to and following exposure to broadcast stimuli	15
Figure 5. Photographs of the six sampled sites in Gainesville, FL	19
Figure 6. Linear regressions depicting the relationships between corticosterone level and chorus density, the proportion of non-calling males, SIL, and body condition in male green treefrogs	23
Figure 7. Corrected circulating corticosterone levels of calling male green treefrogs at sites where: Cuban treefrogs were present and chorusing, Cuban treefrogs were present but not chorusing, and Cuban treefrogs were not present.	23
Figure 8. Corrected circulating corticosterone levels of calling male green treefrogs at six sites sampled in Florida.	24
Figure 9. Linear regressions depicting the relationships between androgens and chorus density, the proportion of non-calling males, SIL, and body condition in male green treefrogs	25
Figure 10. Corrected circulating androgen levels of calling male green treefrogs at sites where: Cuban treefrogs were present and chorusing, Cuban treefrogs were present but not chorusing, and Cuban treefrogs were not present.	25
Figure 11. Corrected circulating androgen levels of calling male green treefrogs at six sites sampled in Florida.	26
Figure 12. Linear regression depicting the relationship between green treefrog density and the proportion of non-calling male green treefrogs	27

**EXAMINING THE POTENTIAL ROLE OF REPRODUCTIVE INTERFERENCE IN  
THE DECLINE OF NATIVE GREEN TREEFROGS FOLLOWING CUBAN  
TREEFROG INVASIONS**

**I. INTRODUCTION**

Invasive species are a leading cause of global declines in native taxa and biodiversity (Clavero and Garcia-Berthou, 2005; Doherty et al., 2016). Introduced species often negatively impact native taxa via direct predation, competitive exclusion, and/or the transmission of novel pathogens (Sakai et al., 2001; Crowl et al., 2008). However, there is also growing evidence implicating reproductive interference in the decline of native taxa following invasions by non-native species (Rhymer and Simberloff, 1996; Uyehara et al., 2007; D'Amore et al., 2009; Kraus, 2015). Reproductive interference refers to instances in which interspecific sexual interactions negatively impact the fitness of at least one of the species involved (Groning and Hochkirch, 2008). For example, European bumblebees (*Bombus terrestris*) and Japanese bumblebees (*Bombus hypocrita*) exhibit similar courtship behaviors, have a high rate of interspecific pairing, but do not produce viable offspring (Kondo et al., 2009). Since Japanese bumblebee queens generally only mate once during their life, mismatings with invasive European bumblebees could pose a threat for native Japanese bumblebee populations (Kondo et al., 2009; see also Uyehara et al., 2007 for a similar example involving introduced Mallard ducks and native Hawaiian ducks).

Here, I examined the potential role of reproductive interference in the context of declines of native green treefrogs (*Hyla cinerea*) following Cuban treefrog (*Osteopilus septentrionalis*)

invasions in the southeastern United States. The Cuban treefrog is native to Cuba, the Cayman Islands, and the Bahamas (Meshaka, 2001). In the early 1920s, Cuban treefrogs were introduced to southern Florida through shipping ports in the lower Florida Keys (Meshaka, 2001). Established breeding populations were documented in Miami soon thereafter (Meshaka, 2001), and have now spread throughout southern and central Florida (Johnson, 2013) and southern Louisiana (Glorioso et al., 2018). Cuban treefrogs are of major conservation concern because they have been linked to population declines and localized extirpation of native anurans as they spread across Florida (Johnson, 2013; Rice et al., 2011; Meshaka, 2001). In particular, Cuban treefrogs have replaced native green treefrogs at sites where they were once common (Johnson, 2013).

How Cuban treefrogs are negatively impacting green treefrogs and other native anurans is unclear (Rice et al., 2011). One prominent hypothesis is that Cuban treefrogs directly prey on native anurans (Meshaka, 2001; Wyatt and Forsys, 2004). However, analysis of stomach contents of 767 adult Cuban treefrogs in South Florida revealed that native frog species were found in only 26 (3.5%) of the frogs examined (Glorioso et al., 2012), suggesting that predation by Cuban treefrogs is not a primary factor driving the decline of native anuran species. There is also little evidence indicating that larval competition is a major factor contributing to the decline of native treefrogs. For instance, there was no detectable effect of Cuban treefrog tadpoles on survival of green treefrog tadpoles in mesocosm experiments (Smith, 2005). The presence of Cuban treefrog tadpoles did, however, result in decreased growth rates and smaller masses at metamorphosis in green treefrogs, but whether these effects translate into population declines is not known (Smith, 2005).

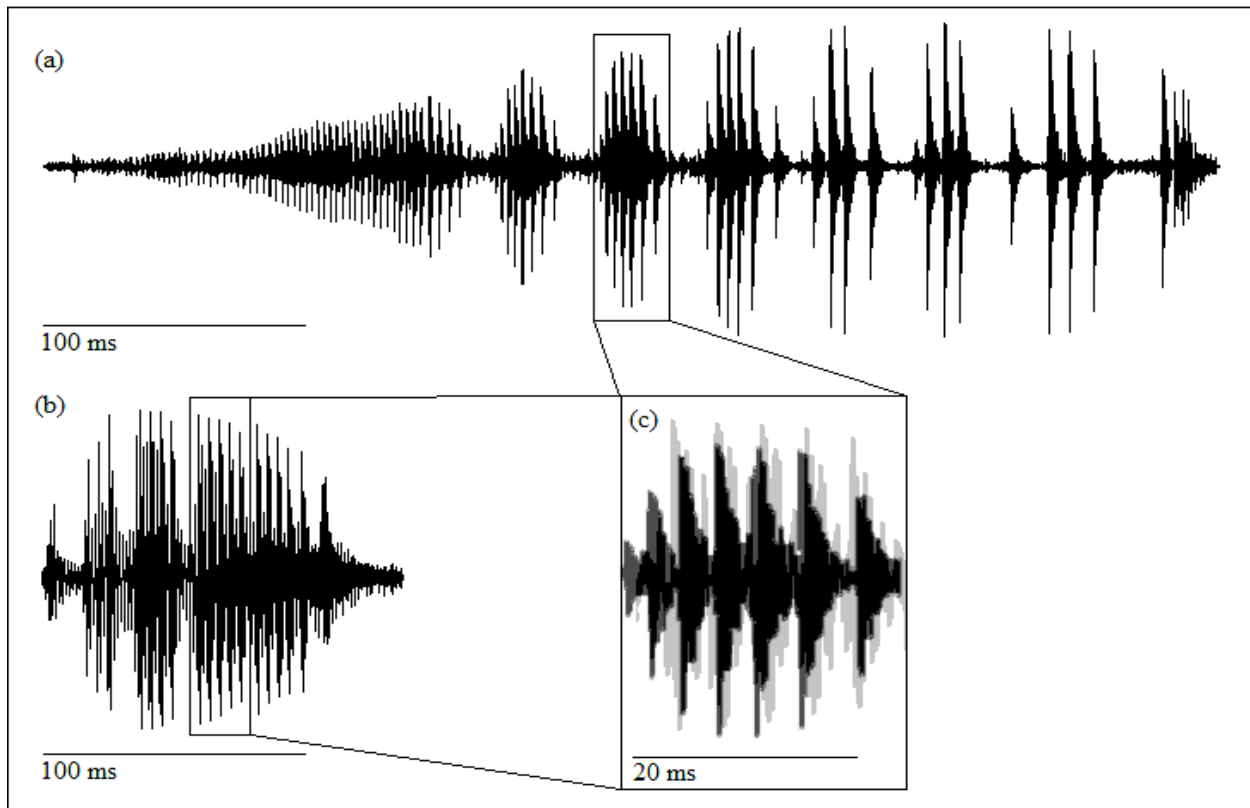
Recent work on Cuban treefrogs and other invasive anurans implicate acoustic interference as an important factor affecting native anuran species (Both and Grant, 2012; Bleach et al., 2015;

Tennessen et al., 2016). Most anurans use acoustic signals to attract mates (Gerhardt and Huber, 2002) and many anurans call in mixed species breeding assemblages, but sympatric species characteristically evolve distinct “channels” (i.e. spectral and temporal characteristics of acoustic signals) that minimize acoustic interference (Chek et al., 2003). The calls of introduced species can, however, overlap and interfere with those of native taxa to alter their behavior. For example, overlap in the calls of Cuban treefrogs and green treefrogs causes male green treefrogs to shorten the duration of their advertisement calls and increase call amplitude when exposed to broadcast Cuban treefrog choruses (Tennessen et al., 2016). Similarly, native Australian marbled frogs (*Limnodynastes convexiusculus*) increase call amplitude and adjust the timing of their advertisement calls to minimize overlap with the vocalizations of invasive cane toads (*Rhinella marina*) (Bleach et al., 2015). In white-banded treefrogs (*Hypsiboas albomarginatus*), males produce shorter call durations with higher spectral peaks in response to broadcast calls of invasive bullfrogs (*Rana catesbeiana*) (Both and Grant, 2012). Because female white-banded treefrogs potentially select mates based on these altered call characteristics, the presence of invasive bullfrog calls could decrease reproductive success in this native treefrog (Both and Grant, 2012).

One aspect of reproductive interference that remains unstudied involves how the courtship signals of invasive species potentially influence the endocrine physiology of native taxa. Advertisement calls of anurans, for example, are known to stimulate the production of gonadal steroids in males and females, which can promote reproductive behaviors (Lynch and Wilczynski, 2006; Chu and Wilczynski, 2001). In contrast, males of many anuran species produce distinct aggressive calls that stimulate the production of glucocorticoids in conspecific male signal receivers (Leary, 2014; Leary and Crocker-Buta, 2018; Leary and Harris, 2013), which suppresses reproductive behavior (Leary et al., 2008a; Leary and Crocker Buta, 2018). In green treefrogs,

dominant males effectively oust competing males from the pool of conspecific males engaged in vocalization during close range aggressive vocal interactions which stimulate glucocorticoid production and suppress vocalization in contest losers, either independently or in conjunction with decreased androgen levels (Leary and Crocker-Buta, 2018; Leary, 2014). In this species, contest losers adopt non-calling alternative “satellite” mating tactics to increase their chances of acquiring mates in the presence of dominant males (Leary and Harris, 2013; Perrill et al., 1978).

Here, I investigated reproductive interference between invasive Cuban treefrogs and native green treefrogs in the context of acoustic modulation of hormone levels because of the remarkable similarities in Cuban treefrog advertisement calls and green treefrog aggressive calls (Fig. 1). For example, in addition to sharing similar carrier frequencies, these two calls are also amplitude modulated (i.e., pulsed) and portions of the Cuban treefrog advertisement call possess similar pulse repetition rates, pulse durations, and pulse rise time characteristics as green treefrog aggressive calls (Fig. 1) – features that are crucial for central auditory processing in anurans (Leary et al., 2008b; Edwards et al., 2003; Rose, et al., 2011, 2015). Similarities in spectral and temporal properties suggest that the advertisement calls of invasive Cuban treefrogs stimulate elevations in circulating glucocorticoid levels in male green treefrogs. Previous vocal playback experiments and hormone manipulation studies provide indirect support for this hypothesis. For example, the reduction in call duration in male green treefrogs in response to broadcast Cuban treefrog choruses (Tennessen et al., 2016) is very similar to the reduction in call duration reported in this species in response to corticosterone (CORT) injections (Leary and Crocker-Buta, 2018). This suggests that alterations in the call properties of green treefrogs in response to broadcast Cuban treefrogs calls could be mediated by elevations in circulating glucocorticoids.



**Figure 1.** Waveform representations of (a) Cuban treefrog advertisement call, (b) green treefrog aggressive call; (c) shows the extent of temporal overlap (■) between the aggressive call of green treefrogs (■) and the advertisement call of Cuban treefrogs (■). In addition to these temporal features, the two calls also have similar spectral components (i.e. both calls have a peak carrier frequency around 2500 Hz, not shown).

Acoustic modulation of circulating hormone levels in green treefrogs by Cuban treefrogs, if it occurs, may be particularly important because the persistence of courtship signaling by Cuban treefrogs (i.e., males produce advertisement calls throughout most of the night, Tennesen et al., 2013) could stimulate chronic elevations in glucocorticoid levels in male green treefrogs. Chronically elevated glucocorticoids can have numerous negative fitness consequences stemming from suppression of reproductive behavior, general wasting, and compromised immunity (Selye, 1936; Christian, 1950; Boonstra and Singleton, 1993; Creel et al., 2013).



I hypothesized that invasive Cuban treefrogs negatively impact green treefrog populations because the courtship calls of Cuban treefrogs stimulate chronic elevations in circulating glucocorticoid levels that suppress reproductive behavior in native green treefrogs. I tested this hypothesis using two approaches. The first approach involved using vocal playback experiments to examine the effects of Cuban treefrog calls on the endocrine physiology of green treefrogs. The second approach involved examining hormone levels and calling behavior of green treefrogs in natural choruses with and without Cuban treefrogs. The combined approaches allowed for determination of whether Cuban treefrog courtship signals cause changes in the endocrine physiology of green treefrogs and whether these effects translate into variation in hormone levels in natural populations of green treefrogs. The second approach, however, also allowed for examination of whether Cuban treefrogs alter the endocrine physiology of green treefrogs via non-acoustically-mediated stimulation of hormone production. For example, invasive species can alter the endocrine physiology of native taxa via visual or chemical cues or through competition for limited food resources (Narayan et al., 2015; Jessop et al., 2015; Santicchia et al., 2018).

# CHAPTER ONE: INVESTIGATING THE EFFECT OF BROADCAST CUBAN TREEFROG CALLS ON HORMONE LEVELS IN NATIVE GREEN TREEFROGS

## I. METHODS

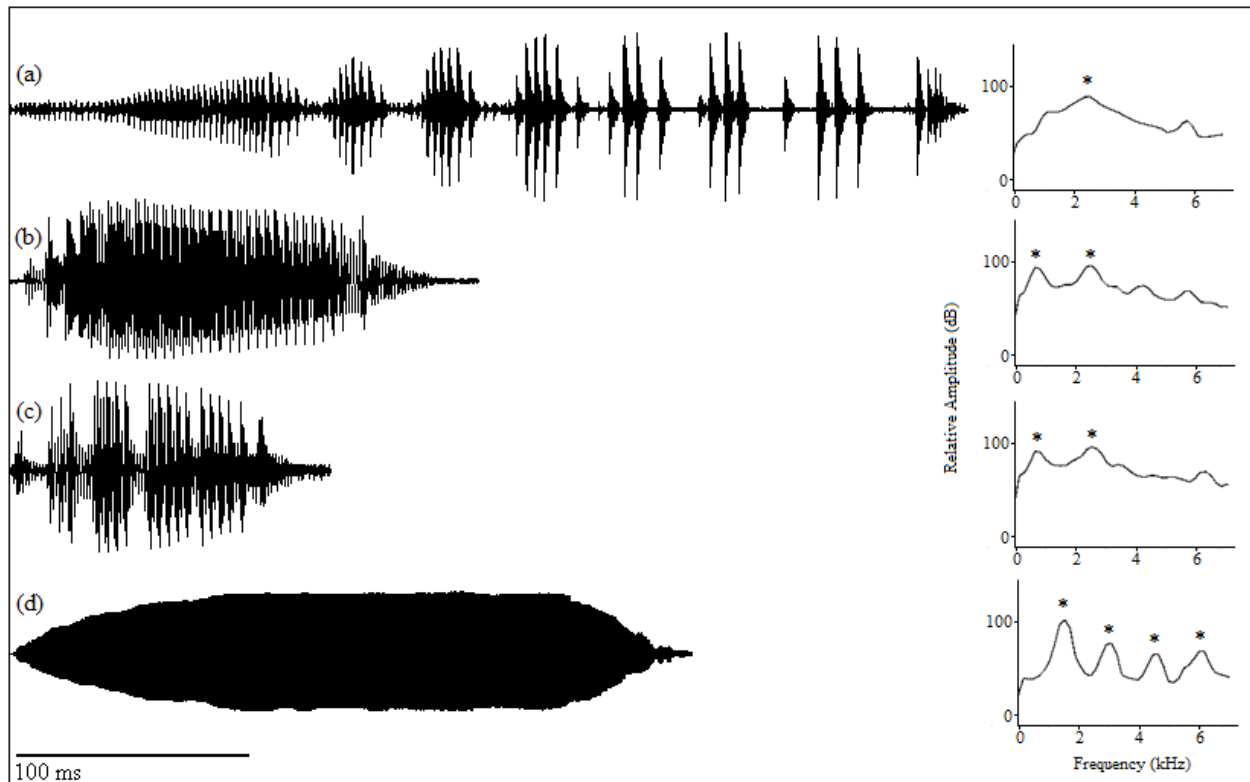
### *General procedures*

Calling male green treefrogs (*Hyla cinerea*) from natural choruses at the University of Mississippi Field Station were used in vocal playback experiments. Green treefrogs in Mississippi are naïve to Cuban treefrog vocalizations, and thus, there has been no opportunity for evolutionary responses to Cuban treefrogs in these populations. Calling male green treefrogs were captured by hand from 2100 to 2300 hrs, when natural choruses were at the highest level of activity. Captured frogs were then placed into individual damp pillow cases and left to acclimate for 1 hr away from the chorus before exposure to the stimuli (see Leary, 2014).

### *Stimuli used in playback experiments*

The playback experiments consisted of five different treatment groups exposed to: 1) Cuban treefrog advertisement calls, 2) green treefrog advertisement calls, 3) green treefrog aggressive calls, 4) pearl spotted owlet (*Glaucidium perlatum*) calls, and 5) silence (Fig. 2). Parameterization of frog calls used in treatment groups 1-3 was based on the analysis of 13 Cuban treefrog advertisement calls, 54 green treefrog advertisement calls, and 24 green treefrog aggressive calls (using Raven software, Cornell Bioacoustics Laboratory, Ithaca, NY, U.S.A.). Call duration and carrier frequency showed the most variation among these three frog call types.

To account for the variation of calls in the playback experiments and to avoid pseudo-replication (Kroodsma, 1989), three representative calls were selected and used in each treatment group (Table 1). The first call possessed the mean characteristics for call duration and carrier frequency. The second call possessed call parameters with a call duration 1 standard deviation above the mean and a carrier frequency 1 standard deviation below the mean. The third call possessed call parameters with a call duration 1 standard deviation below the mean and a carrier frequency 1 standard deviation above the mean. The pearl spotted owlet call and silence acted as controls in the playback experiments. The pearl spotted owlet, native to Africa, is a natural predator of anurans (Sinclair and Ryan, 2009). The owl call thus acted as a novel stimulus predator signal. To account for the variation of calls and to avoid pseudo-replication, three pearl spotted owlet vocalizations that varied in carrier frequencies were used in this treatment group (see Table 1). Calls were obtained from the following sources: mean frequency – xeno-canto, CAT# 266417; low frequency – Van Wyk, 2013; high frequency – Macaulay Library, CAT# ML21585441.



**Figure 2.** Waveform representations of the signals used in playback experiments: (a) Cuban treefrog advertisement call, (b) green treefrog advertisement call, (c) green treefrog aggressive call, and (d) pearl spotted owlet call. To the right of each waveform are spectra depicting peak carrier frequencies (\*) for the calls.

**Table 1.** Call durations and peak carrier frequencies for all acoustic stimuli used in playback experiments. Cuban treefrog advertisement calls had one peak carrier frequency, green treefrog advertisement and aggressive calls had two peak carrier frequencies, and pearl spotted owlet calls had four peak carrier frequencies (see Fig. 2). “High” and “low” call designations refer to 1 SD above and below the mean for that call parameter.

<b>Cuban treefrog advertisement call</b>	<b>Peak carrier frequency (Hz)</b>	<b>Call duration (ms)</b>
High call	2577	450
Mean call	2420	487
Low call	2196	552
<b>Green treefrog advertisement call</b>	<b>Peak carrier frequencies (Hz)</b>	<b>Call duration (ms)</b>
High call	874, 2936	128
Mean call	717, 2555	139

Low call	672, 2286	178
<b>Green treefrog aggressive call</b>	<b>Peak carrier frequencies (Hz)</b>	<b>Call duration (ms)</b>
High call	941, 2756	137
Mean call	873, 2689	142
Low call	728, 2622	153
<b>Pearl spotted owlet call</b>	<b>Peak carrier frequencies (Hz)</b>	<b>Call duration (ms)</b>
High call	2039, 4011, 6073, 8090	572
Mean call	1546, 3048, 4571, 6095	299
Low call	1389, 2779, 4235, 5535	301

#### *Exposure to the broadcast stimuli*

After one hour of acclimation, individuals were sampled for blood via cardiac puncture (in under 2 min) with a heparinized hypodermic needle (see Leary, 2014) and then placed back into separate pillowcases and acoustic chambers. Each acoustic chamber was constructed by lining a 13” fabric cube storage bin with 0.125” Sound Transmission Class 27 soundproofing material sheet (Soundproof Cow, Chambersburg, PA, U.S.A.) and 2.5” Noise Reduction Coefficient 0.6 eggcrate foam (Foam Factory, Macomb, MI, U.S.A.). Individuals were randomly exposed to one of the five treatments at 90 db SPL for 45 min with a Bluetooth speaker (Anker Soundcore, Shenzhen, China). The time period for exposure to broadcast anuran calls (45 minutes) is ecologically relevant because males in natural choruses produce aggressive calls for at least 45 minutes at the onset of nightly chorus activity and advertisement calls of both species are produced for several hours each night (Leary, 2014).

All stimuli were broadcast for an equivalent amount of time and at a 1:3 stimulus:silence ratio to control for total acoustic energy. Following exposure to the treatment, a second blood

sample was immediately taken. Blood samples were then stored on ice until they were taken back to the lab (<4 hrs). Blood was centrifuged at 2500 RPM for 12 min to separate plasma and subsequently frozen at -20°C until radioimmunoassay was done to quantify hormone levels. Circulating hormone levels were measured from 9 males exposed to Cuban treefrog advertisement calls (high=2, mean=3, low=4), 10 males exposed to green treefrog advertisement calls (high=2, mean=3, low=5), 8 males exposed to green treefrog aggressive calls (high=0, mean=4, low=4), 12 males exposed to pearl spotted owlet calls (high=5, mean=3, low=4), and 10 males exposed to silence.

Previous work has shown that the magnitude of the CORT response to acoustic signals is inversely correlated with individual body size (Leary, 2014). In addition, circulating CORT levels are negatively correlated with individual body condition and circulating androgen levels are positively correlated with individual body condition (Leary and Harris, 2013). Hence, all individuals were measured from the tip of the snout to the end of the ischium (e.g., snout-ischial length, SIL) and weighed after obtaining the second blood sample. Body condition was calculated by obtaining the residual values from a linear regression of the cubed root body mass on SIL and dividing those values by SIL (Leary et al., 2015).

#### *Column Chromatography and Radioimmunoassay*

All blood samples (n=98, 49 pre-stimulus and 49 post-stimulus) were examined for levels of dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT). Hormone separation and quantification of hormone levels were done using column chromatography and radioimmunoassay following the methods described in Leary (2014). Briefly, plasma samples were incubated overnight with radiolabeled 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., #20R-TR018W) and used for both T and DHT assays. CORT antibody was purchased from MP Biomedicals, LLC (Solon, OH, U.S.A., #07120016). All samples were assayed in duplicate.

Plasma samples from males in the playback experiments were analyzed for DHT, T, and CORT levels in three assays. Samples were randomized among assays but pre- and post-stimulus exposure blood samples from the same individuals were always included in the same assay. Mean intra-assay coefficients of variation for DHT, T, and CORT were 22%, 14%, and 11%, respectively, based on four standards run with each assay. Inter-assay coefficients of variation for DHT, T, and CORT were 2%, 14%, and 5.2%, respectively.

### *Statistical Analysis*

Analysis of variance (ANOVA) was first used to determine if body size or body condition differed between treatment groups. Repeated measures ANOVAs were used to determine if the results from the three representative calls in each treatment group could be combined for the remainder of the analysis based on comparisons of pre- and post-stimulus CORT and total androgen (DHT + T) levels. Next, I compared the effects of the treatments on pre-stimulus and

post-stimulus CORT and total androgen (DHT + T) levels using a repeated measures ANOVA. I then used a paired t-test to examine CORT and total androgen levels prior to and after exposure to the broadcast stimuli.

## II. RESULTS

### *Body condition and size*

Body size did not differ among the treatment groups ( $F_4=1.209$ ,  $p=0.320$ ), nor did body condition ( $F_4=1.676$ ,  $p=0.173$ ). Since body size and condition did not differ between treatments, they were excluded from the remainder of the analysis.

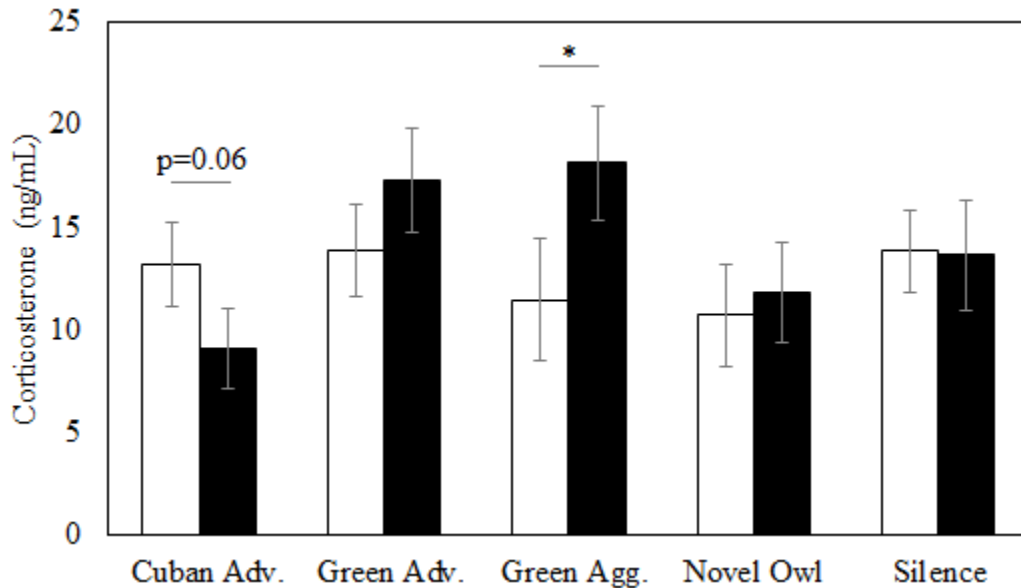
### *Corticosterone levels*

There was no interaction among the three representative calls in each treatment and CORT production for any of the broadcast stimuli (Cuban treefrog advertisement calls  $F_{2,7}=1.846$ ,  $p=0.227$ ; green treefrog advertisement calls  $F_{2,6}=2.693$ ,  $p=0.146$ ; green treefrog aggressive calls  $F_{1,6}=0.073$ ,  $p=0.796$ ; and pearl spotted owlet calls  $F_{2,9}=0.793$ ,  $p=0.482$ ). The results from the three representative calls in each treatment group were thus combined for the remainder of the analysis.

When averaged across treatment groups, there was no overall change in CORT levels ( $F_{1,44}=1.835$ ,  $p=0.182$ ). However, the change in CORT levels did differ among treatment groups, resulting in a significant treatment by time interaction ( $F_{4,44}=2.909$ ,  $p=0.032$ ). Males that were exposed to green treefrog aggressive calls showed a significant increase in CORT ( $t_7=-4.614$ ,  $p=0.002$ ) (Fig. 3). There was no significant change in CORT levels for males exposed to green treefrog advertisement calls ( $t_8=-1.116$ ,  $p=0.297$ ), pearl spotted owlet calls ( $t_{11}=-0.546$ ,  $p=0.596$ ),



or silence ( $t_9=0.091$ ,  $p=0.930$ ). Males exposed to Cuban treefrog advertisement calls actually showed a marginal decrease in CORT ( $t_9=2.173$ ,  $p=0.06$ ) (Fig. 3).



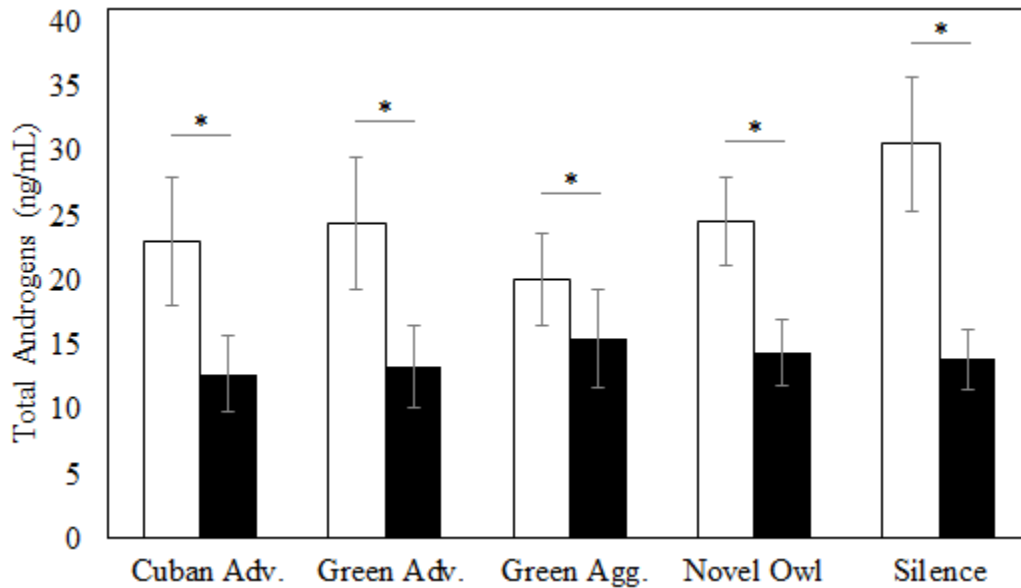
**Figure 3.** Corticosterone levels prior to (□) and after (■) exposure to broadcast Cuban treefrog advertisement calls (n=9), green treefrog advertisement calls (n=10), green treefrog aggressive calls (n=8), novel pearl spotted owlet calls (n=12), and silence (n=10).

### *Total Androgen Levels*

There was no interaction among the three representative calls in each treatment and androgen levels for any of the broadcast stimuli (Cuban treefrog advertisement calls  $F_{2,7}=2.078$ ,  $p=0.196$ ; green treefrog advertisement calls  $F_{2,6}=3.146$ ,  $p=0.116$ ; green treefrog aggressive calls  $F_{1,6}=3.529$ ,  $p=0.109$ ; and pearl spotted owlet calls  $F_{2,9}=0.608$ ,  $p=0.565$ ). The results from the three representative calls in each treatment group were thus combined for the remainder of the analysis.

Averaged across all stimulus types, there was an overall decrease in pre- to post-stimulus androgen levels ( $F_{1,44}=82.067$ ,  $p<0.001$ ): Cuban treefrog advertisement calls ( $t_9=4.368$ ,  $p=0.002$ ), green treefrog advertisement calls ( $t_8=4.788$ ,  $p=0.001$ ), green treefrog aggressive calls ( $t_7=3.172$ ,

p=0.016), pearl spotted owlet call ( $t_{11}=5.761$ ,  $p<0.001$ ), and silence ( $t_9=4.144$ ,  $p=0.003$ ) (Fig. 4). In addition, there was an interaction between treatment and pre- and post-stimulus androgen levels among the treatment groups ( $F_{4,44}=2.577$ ,  $p=0.050$ ); this was attributable to lower pre-stimulus androgen levels among individuals exposed to green treefrog aggressive calls than those among individuals exposed to silence.



**Figure 4.** Total Androgen (T + DHT) levels prior to (□) and after (■) exposure to broadcast Cuban treefrog advertisement calls (n=9), green treefrog advertisement calls (n=10), green treefrog aggressive calls (n=8), novel pearl spotted owlet calls (n=12), and silence (n=10).

### III. DISCUSSION

Despite striking similarities in the spectral and temporal properties of Cuban treefrog advertisement calls and green treefrog aggressive calls, playback experiments revealed no evidence that the calls of Cuban treefrogs stimulate elevations in circulating glucocorticoids in green treefrogs. In fact, the Cuban advertisement call was the only stimulus that led to a marginal

decrease in glucocorticoid levels. The only treatment that elicited an increase in glucocorticoids was the green treefrog aggressive call, which is consistent with previous research (Leary, 2014).

It is unclear why Cuban treefrog advertisement calls do not elicit elevations in circulating glucocorticoids. One possibility lies in the neural processing of pulse repetition rate by auditory neurons in anurans (Leary et al., 2008b; Edwards et al., 2003; Rose, et al., 2011, 2015). For example, midbrain auditory neurons of Pacific treefrogs (*Pseudacris regilla*) only respond to a specific pulse repetition rate; when the pulse repetition rate is too low or too high it can actually hyperpolarize the cell (Edwards et al., 2007). This is important because, unlike the stereotyped pulse repetition rate of the advertisement calls of many anurans, the pulse repetition rate of Cuban treefrog advertisement calls varies temporally. Hence, non-similar portions of the Cuban treefrog advertisement call could hyperpolarize the auditory neurons that may be involved in triggering endocrine responses. If the neuron is hyperpolarized by various preceding elements of the call, the portion of the Cuban treefrog advertisement call that is similar to the green treefrog aggressive call may not be sufficient to depolarize the cell.

All treatments showed a significant decrease in total androgens (testosterone and dihydrotestosterone) after being exposed to the stimuli. This response is likely due to the period of acclimation (1 hr) before the playback experiment. During this period, each individual was placed in a damp pillow away from the chorus. Conspecific chorus sounds are known to stimulate androgen production in male green treefrogs (Burmeister and Wilczynski, 2000). Hence, the period of relative silence during acclimation may have resulted in decreased stimulation by chorus sounds that led to decreased androgen levels across all treatment groups.

Overall, my results suggest that exposure to a single Cuban treefrog call does not elicit an increase in glucocorticoids in green treefrogs. However, how circulating glucocorticoid and

androgen levels of male green treefrogs are potentially altered by choruses of Cuban treefrogs is not known. This is important because the endocrine response to isolated calls and more complex chorus sounds may be very different in anurans. For example, in green treefrogs, conspecific choruses stimulate androgen production but broadcast representations of single advertisement calls do not (Burmeister and Wilczynski, 2000; Leary, 2014). Moreover, the presence of Cuban treefrogs could alter the endocrine physiology of green treefrogs through visual cues or competition for limited resources (Narayan et al., 2015). For example, invasive cane toads (*Rhinella marina*) cause an increase in glucocorticoid levels in native Fiji ground frogs (*Platymantis vitianus*) when placed together in large (4 x 10,000 m<sup>2</sup>) enclosures, presumably because toads are visually threatening to ground frogs or because direct competition for food elicits a stress response (Narayan et al., 2015). In addition, prolonged exposure to invasive cane toads was correlated with lower body condition and smaller egg clutch sizes in the Fiji ground frogs (Narayan et al., 2015), suggesting that the presence of alien cane toads can lead to declines in Fiji ground frog populations via effects of elevated glucocorticoids. Because invasive Cuban treefrogs are known predators of other anurans (Meshaka, 2001), it is entirely possible that visual, rather than auditory, stimulation elicits a stress response in green treefrogs. Alternatively, competition between the two species for limited resources could stimulate elevation in glucocorticoid levels in native green treefrogs. To address these possibilities, Chapter Two examines the endocrine physiology of green treefrogs in natural populations and in the presence and absence of Cuban treefrogs.

**CHAPTER TWO: HORMONE LEVELS AND BEHAVIOR OF GREEN TREEFROGS  
IN NATURAL CHORUSES WITH AND WITHOUT CUBAN TREEFROGS**

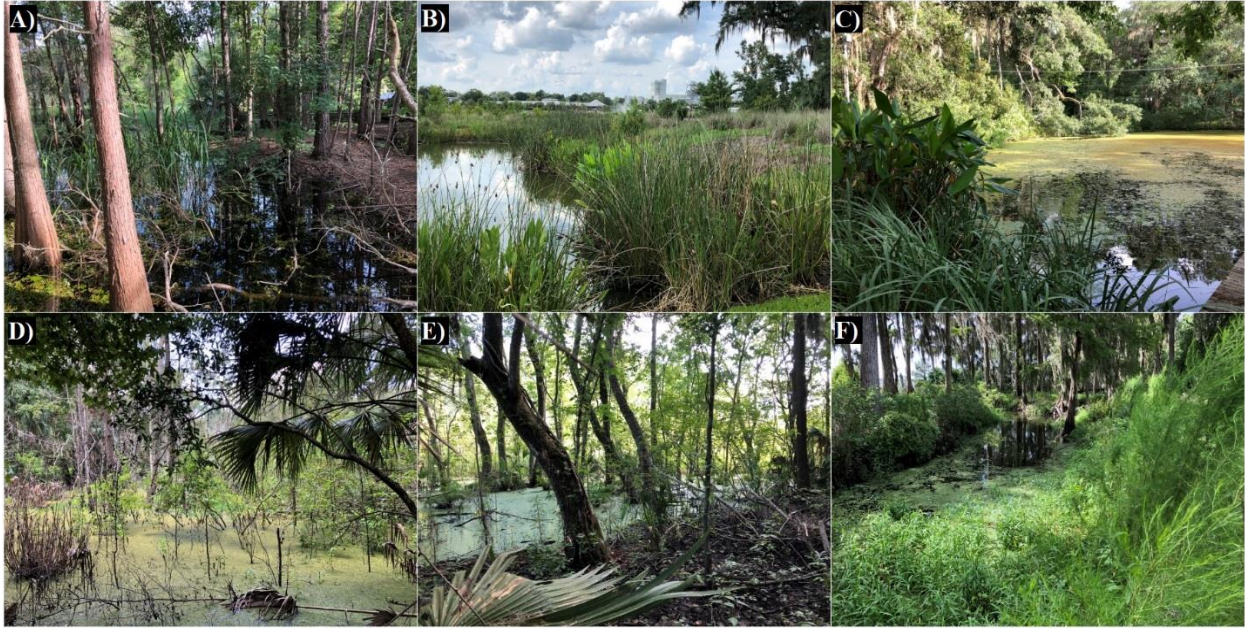
**I. METHODS**

*Site selection in Gainesville, FL*

I compared circulating levels of glucocorticoids and androgens in male green treefrogs (*Hyla cinerea*) and the proportion of calling and non-calling male green treefrogs at ponds in central Florida where Cuban treefrogs were present (“Cuban +” sites) and absent (“Cuban –” sites). Sampling occurred throughout June and July during the breeding season for both green treefrogs and Cuban treefrogs. A total of 6 sites were used that included 3 Cuban + sites and 3 Cuban – sites in Gainesville, FL (Table 2, Fig. 5).

**Table 2.** Sites and GPS coordinates where green treefrogs were sampled in Gainesville, FL.

<b>Cuban Present Sites</b>	<b>Site ID</b>	<b>GPS Coordinates</b>
Natural Area Teaching Lab (University of Florida)	Cuban+1	29°38'01.0"N 82°22'02.3"W
Depot Park	Cuban+2	29°38'37.7"N 82°19'17.1"W
Private pond	Cuban+3	29°39'52.8"N 82°22'49.7"W
<b>Cuban Absent Sites</b>	<b>Site ID</b>	<b>GPS Coordinates</b>
Private wetland	Cuban–1	29°35'38.6"N 82°20'40.3"W
Lake Alice wetland (University of Florida)	Cuban–2	29°38'32.0"N 82°21'39.3"W
Newnan’s Lake private wetland	Cuban–3	29°40'07.3"N 82°14'17.0"W



**Figure 5.** Photographs of the six sites where green treefrogs were sampled. Cuban + sites: (A) Cuban+1, (B) Cuban+2, and (C) Cuban+3. Cuban – sites: (D) Cuban–1, (E) Cuban–2, and (F) Cuban–3.

### *General sampling procedures*

Sampling took place between 2200 and 2400 hrs, when natural choruses were at the highest level of activity. Male green treefrogs at Cuban – sites were sampled once. In contrast, male green treefrogs at Cuban + sites were sampled twice: once when Cuban treefrogs were chorusing (Cuban + Call) and once when Cuban treefrogs were present but not chorusing (Cuban + No Call). Sampling green treefrogs in the absence and presence of chorusing Cuban treefrogs allowed me to examine how circulating glucocorticoid or androgen levels in green treefrogs potentially vary in the mere presence of Cuban treefrogs versus when Cuban treefrogs are vocalizing.

Beginning at 2100 hrs, I performed visual and auditory surveys to get an estimate of the proportion of non-calling to calling male green treefrogs at each site. Next, I measured the population density of green treefrogs. Conspecific chorus density is known to be positively correlated with circulating glucocorticoid levels in other anuran species (Leary et al., 2008c).

Density was calculated at each site by randomly selecting a calling individual and counting the number of conspecific males in a 1m radius. This was repeated 5 times with 5 different focal individuals over 20 min to obtain an average density (number of calling or non-calling males per 1m<sup>2</sup>). After the population density was measured, I captured and rapidly (in under 2 min) obtained blood samples via cardiac puncture from calling and non-calling male green treefrogs between 2200 and 2400 hrs for hormone analysis. Individuals were then measured from the tip of the snout to the end of the ischium (e.g., snout-ischial length, SIL) and weighed. Body condition was then calculated by obtaining the residual values from a linear regression of the cubed root body mass on SIL and dividing those values by SIL (Leary et al., 2015).

#### *Column Chromatography and Radioimmunoassay*

All blood samples (n=132) were assayed for levels of dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT) (see methods in Chapter One).

Plasma samples were analyzed and randomized in two assays. Mean intra-assay coefficients of variation for DHT, T, and CORT were 22.5%, 14.5%, and 14.5%, respectively, based on four standards run with both assays. Inter-assay coefficients of variation for DHT, T, and CORT were 12.5%, 6%, and 5%, respectively.

#### *Statistical Analysis*

ANOVA was first used to determine if CORT levels, total androgen levels (DHT + T), body size, and body condition differed between calling and non-calling male green treefrogs. I then examined the relationship between hormone levels of calling male green treefrogs and several variables that could influence circulating hormone levels (body size, body condition, conspecific

density, and the proportion of non-calling male green treefrogs) using a multiple linear regression. I used scatterplots to visualize pairwise relationships between hormone levels and body size, body condition, conspecific density, and proportion of non-calling male green treefrogs.

To control for the effects of these variables on hormone levels, I used the residuals from the multiple regression in place of raw hormone levels to determine whether hormone levels of calling male green treefrogs varied in the presence and absence of Cuban treefrogs. Specifically, I examined how hormone levels, corrected for these variables, differed among Site Type (Cuban –, Cuban + Call, and Cuban + No Call) and individual sites nested within Site Type. Individual sites were nested into Site Type to determine if and how hormone levels varied among Site Types and to account for random variation among sites.

ANOVA was used to test for differences between Site Type (Cuban –, Cuban + Call, and Cuban + No Call) and green treefrog density. ANOVA was also used to test for differences between Site Type and proportion of non-calling male green treefrogs. The relationship between green treefrog density and the proportion of non-calling male green treefrogs was examined using linear regression.

## **II. RESULTS**

### *Differences in calling and non-calling male green treefrogs*

Calling male green treefrogs had significantly higher circulating total androgen levels than non-calling males ( $F_{1,130}=17.080$ ,  $p<0.001$ ; mean  $\pm$  SE, callers (n=116):  $27.5 \pm 1.32$  ng/ml, non-callers (n=16):  $12.4 \pm 1.89$  ng/ml), but the two groups of males did not differ in circulating CORT levels ( $F_{1,130}=0.175$ ,  $p=0.676$ ; mean  $\pm$  SE, callers (n=116):  $2.5 \pm 0.35$  ng/ml, non-callers (n=16):  $2.4 \pm 0.67$  ng/ml). In addition, calling male green treefrogs were significantly larger ( $F_{1,130}=11.656$ ,

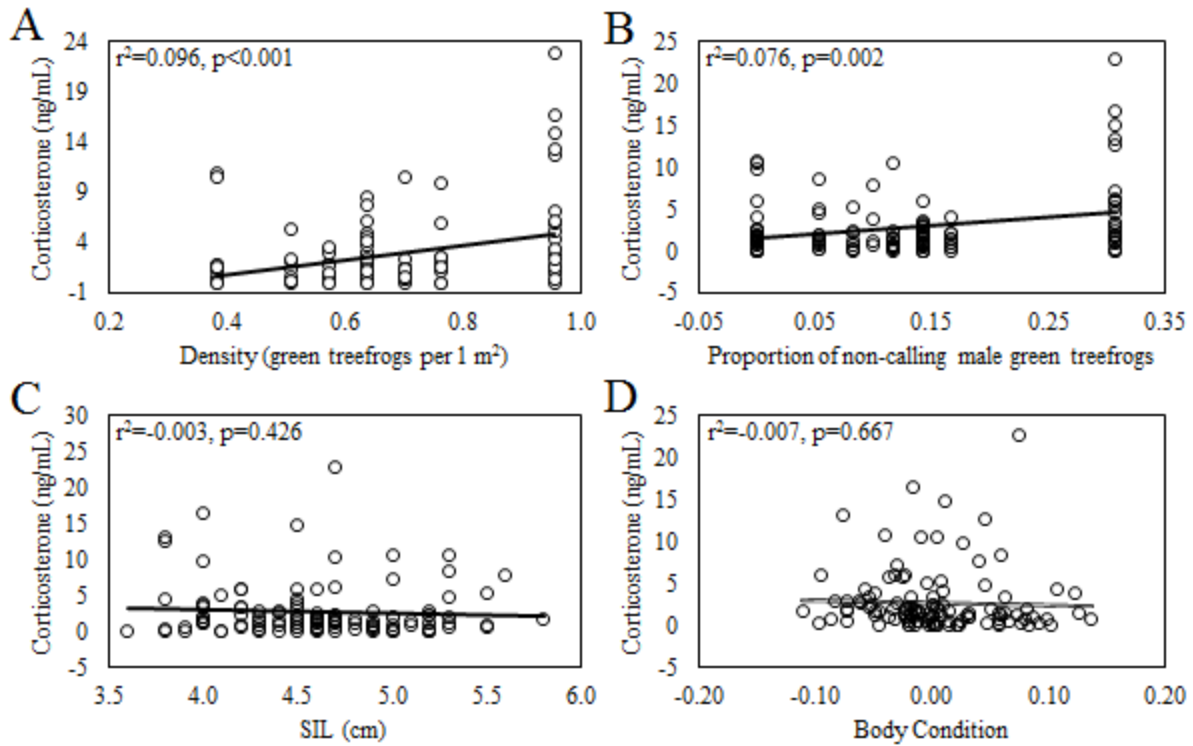


$p=0.001$ ; mean  $\pm$  SE, callers ( $n=116$ ):  $4.6 \pm 0.04$  cm, non-callers ( $n=16$ ):  $4.1 \pm 0.11$  cm) and were in better body condition than non-calling males ( $F_{1,130}=4.160$ ,  $p=0.043$ ; mean  $\pm$  SE, callers ( $n=116$ ):  $0.003 \pm 0.005$ , non-callers ( $n=16$ ):  $-0.025 \pm 0.013$ ). Because of the relatively small sample sizes for non-calling males and differences in these parameters for non-calling and calling males, only calling male green treefrogs were used in the remainder of the analysis.

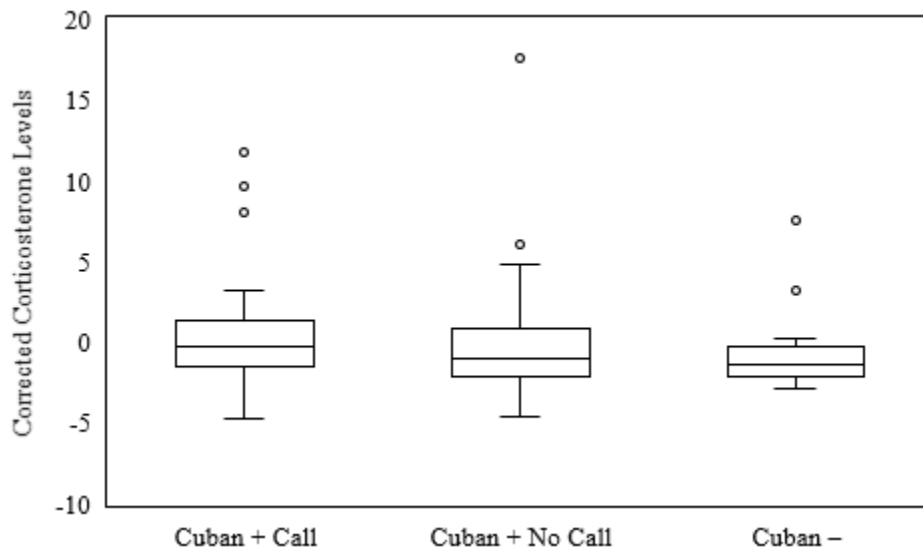
### *Circulating glucocorticoid levels of calling male green treefrogs*

Multiple regression results indicated that body size, body condition, conspecific density, and proportion of non-calling male green treefrogs were significant predictors of circulating CORT levels in male green treefrogs ( $F_{4,109}=3.554$ ,  $r^2=0.083$ ,  $p=0.009$ ). There was a significant positive relationship between CORT and green treefrog density ( $F_{1,114}=12.938$ ,  $r^2=0.096$ ,  $p<0.001$ ) and CORT and proportion of non-calling male green treefrogs ( $F_{1,114}=10.506$ ,  $r^2=0.076$ ,  $p=0.002$ ) (Fig. 6a, 6b). There was no significant relationship between CORT and SIL ( $F_{1,114}=0.639$ ,  $r^2=-0.003$ ,  $p=0.426$ ) or CORT and body condition ( $F_{1,114}=0.186$ ,  $r^2=-0.007$ ,  $p=0.667$ ) (Fig. 6c, 6d).

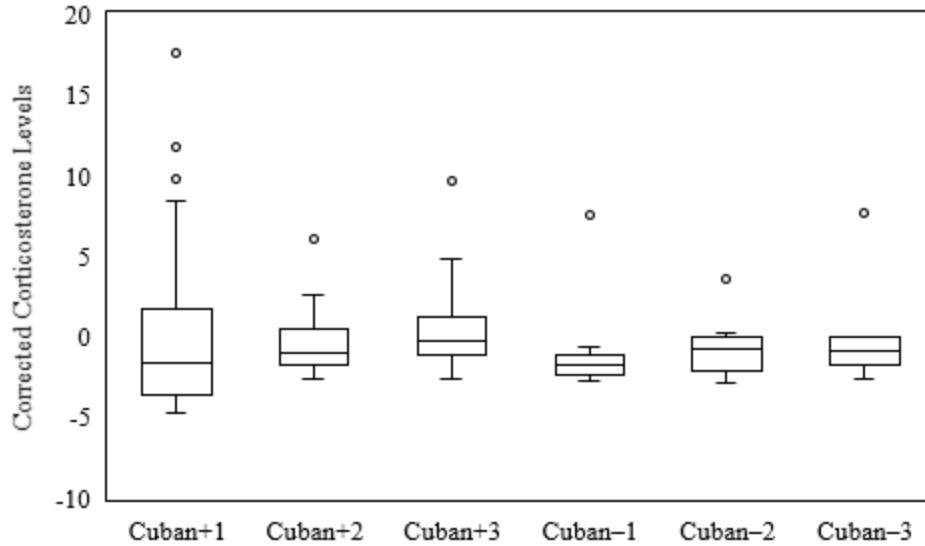
After controlling for the effects of these variables by using the residuals obtained from multiple regression, there was no evidence that circulating CORT levels in male green treefrogs differed among sites where Cuban treefrogs were absent, present and chorusing, or present but not chorusing ( $F_{2,6(\text{Site Type})}=2.094$ ,  $p=0.202$ ) (Fig. 7). In addition, there was no difference in circulating CORT levels in male green treefrogs among individual sites ( $F_{2,6(\text{Site}(\text{Site Type}))}=0.823$ ,  $p=0.555$ ) (Fig. 8).



**Figure 6.** Linear regressions depicting the relationships between A) corticosterone and green treefrog density, B) corticosterone and proportion of non-calling male green treefrogs, C) corticosterone and snout-ischial length (SIL), and D) corticosterone and body condition.



**Figure 7.** Corrected circulating corticosterone levels of calling male green treefrogs at sites where Cuban treefrogs were present and chorusing (Cuban + Call), Cuban treefrogs were present but not chorusing (Cuban + No Call), and Cuban treefrogs were absent (Cuban -). Corticosterone levels were corrected using the residuals from multiple linear regression (see text).



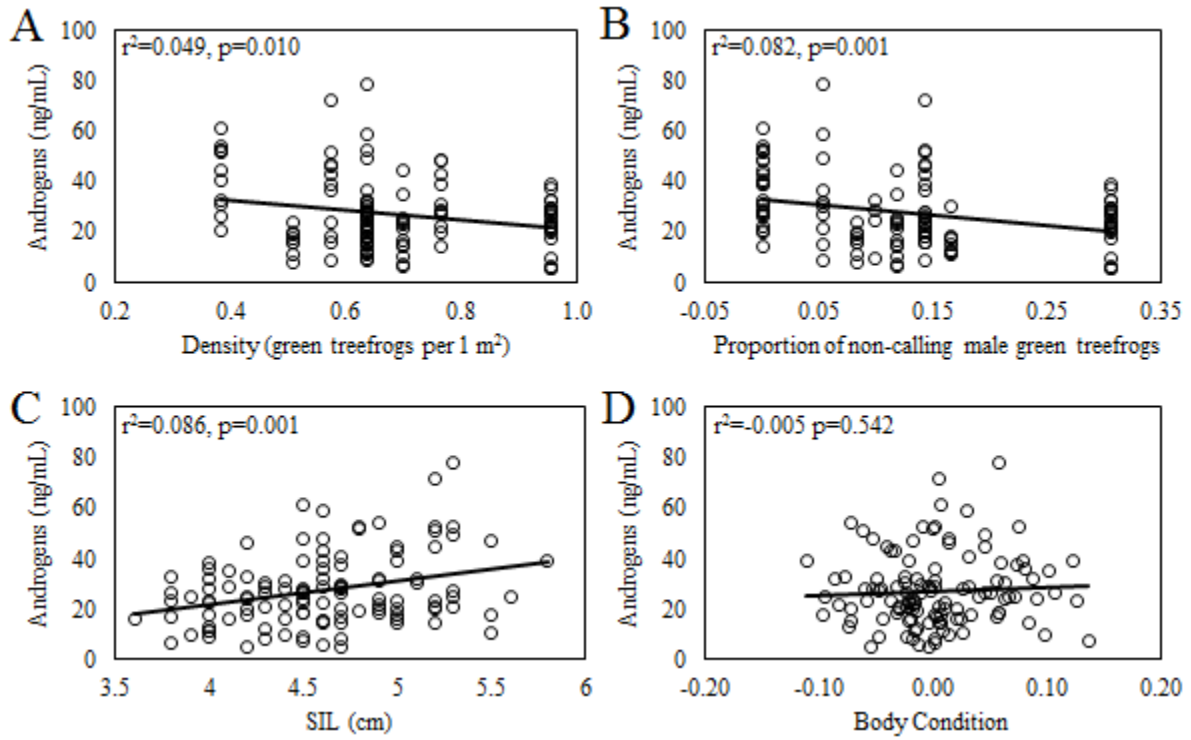
**Figure 8.** Corrected circulating corticosterone levels of calling male green treefrogs at the six sites sampled in Gainesville, FL. Corticosterone levels were corrected using the residuals from multiple linear regression (see text).

*Circulating androgen levels of calling male green treefrogs*

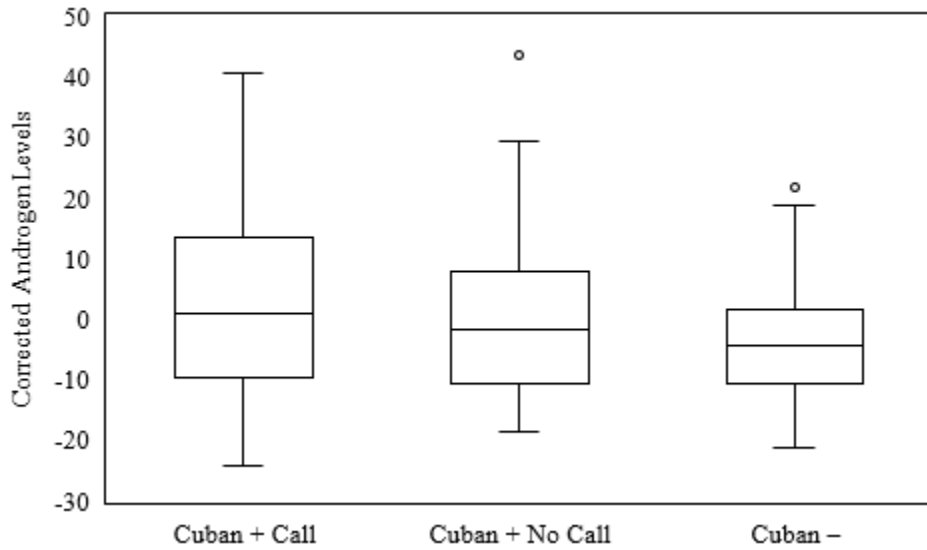
Multiple regression results indicated that body size, body condition, conspecific density, and proportion of non-calling male green treefrogs were significant predictors of circulating androgen levels in male green treefrogs ( $F_{4,109}=3.948$ ,  $r^2=0.095$ ,  $p=0.005$ ). There was a significant negative relationship between androgens and green treefrog density ( $F_{1, 114}=6.828$ ,  $r^2=0.049$ ,  $p=0.010$ ) and androgens and proportion of non-calling male green treefrogs ( $F_{1, 114}=11.204$ ,  $r^2=0.082$ ,  $p=0.001$ ) (Fig. 9a, 9b). There was a significant positive relationship between androgens and SIL ( $F_{1, 114}= 11.864$ ,  $r^2=0.086$ ,  $p=0.001$ ) (Fig. 9c). There was no relationship between androgens and body condition ( $F_{1, 114}=0.375$ ,  $r^2=-0.005$   $p=0.542$ ) (Fig. 9d).

After controlling for the effects of these variables by using the residuals obtained from multiple regression, there was no evidence that circulating androgen levels in male green treefrogs differed among sites where Cuban treefrogs were absent, present and chorusing, or present but not chorusing ( $F_{2,6(\text{Site Type})}=1.172$ ,  $p=0.371$ ) (Fig. 10). In addition, there was no difference in

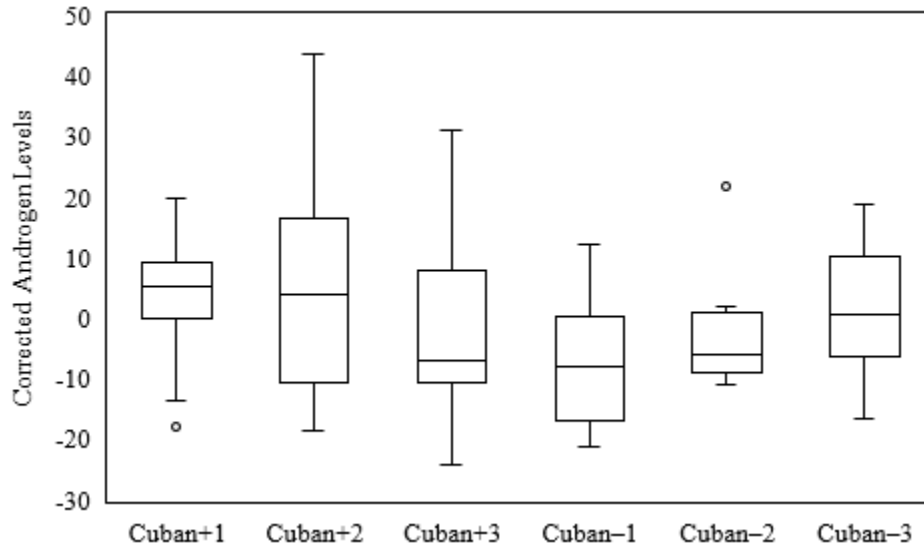
circulating androgen levels in male green treefrogs among individual sites ( $F_{2,6(\text{Site}(\text{Site Type}))}=2.013$ ,  $p=0.070$ ) (Fig. 11).



**Figure 9.** Linear regressions depicting the relationships between A) androgens and green treefrog density, B) androgens and proportion of non-calling male green treefrogs, C) androgens and snout-ischial length (SIL), and D) androgens and body condition.



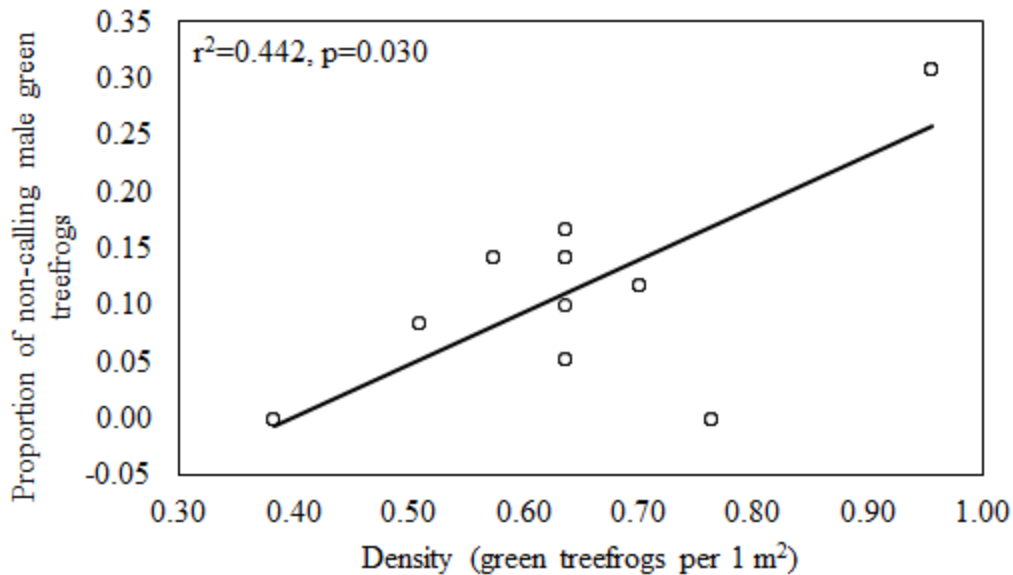
**Figure 10.** Corrected circulating androgen levels of calling male green treefrogs at sites where Cuban treefrogs were present and chorusing (Cuban + Call), Cuban treefrogs were present but not chorusing (Cuban + No Call), and Cuban treefrogs were absent (Cuban -). Androgen were corrected using the residuals from multiple linear regression (see text).



**Figure 11.** Corrected circulating androgen levels of calling male green treefrogs at the six sites sampled in Gainesville, FL. Androgen levels were corrected using the residuals from multiple linear regression (see text).

*Proportion of non-calling green treefrogs and density*

The proportion of non-calling male green treefrogs did not differ between sites where Cuban treefrogs were present and absent ( $F_{2,6}=0.530$ ,  $p=0.614$ ). Similarly, the density of male green treefrogs did not differ between sites where Cuban treefrogs were present and absent ( $F_{2,6}=0.099$ ,  $p=0.908$ ). However, there was a significant positive correlation between the density of green treefrogs and the proportion of non-calling male green treefrogs ( $F_{1,7}=7.345$ ,  $r^2=0.442$ ,  $p=0.030$ ) (Fig. 12).



**Figure 12.** Linear regression depicting the correlation between green treefrog density and proportion of non-calling male green treefrogs.

### III. DISCUSSION

Results suggest that the presence of Cuban treefrogs is not correlated with the levels of circulating glucocorticoids and androgens in native green treefrogs. Furthermore, whether Cuban treefrogs were vocalizing or present and not vocalizing was unrelated to circulating hormone levels in green treefrogs. These results are consistent with the results obtained from playback experiments indicating that the vocalizations of Cuban treefrogs do not elicit a stress response in male green treefrogs. In addition, these results indicate Cuban treefrogs are not influencing circulating glucocorticoid or androgen levels of green treefrogs, suggesting that visual cues, olfactory cues, or interspecific competition from Cuban treefrogs are unlikely to modulate the endocrine physiology of green treefrogs.

I found that body size, body condition, conspecific density, and proportion of non-calling male green treefrogs were strong predictors for circulating glucocorticoid and androgen levels in green treefrogs. In addition, conspecific density and proportion of non-calling male green treefrogs were positively correlated with one another. This pattern has been seen in other anuran species as

well (Leary et al., 2008c; Ovaska and Hunte 1992). For example, chorus density in Woodhouse's toads (*Bufo woodhousii*) was positively correlated with the proportion of non-calling satellite males (Leary et al., 2008c). Moreover, chorus density in this species was positively correlated with glucocorticoid levels (Leary, et al., 2008c). Similarly, I found that corticosterone levels in green treefrogs are positively correlated with both chorus density and the proportion of non-calling males. This relationship potentially occurs because a higher density of conspecifics is expected to increase the frequency of aggressive interactions, and high glucocorticoid levels mediate the adoption of non-calling alternative mating tactics. Hence, under dense chorus conditions more males will abandon calling behavior. I also found that circulating androgen levels were negatively correlated with the chorus density of green treefrogs. Similarly, increased aggressive interactions in more dense choruses may be driving this relationship despite evidence that chorus sounds can stimulate androgen production (Burmeister and Wilczynski, 2000).

It is worth noting that I did not observe any interactions between adult Cuban treefrogs and adult green treefrogs while sampling the sites. This was because at all the Cuban + sites, Cuban treefrogs and green treefrogs were largely partitioned from one another when vocalizing. For instance, Cuban treefrogs vocalized high in the trees, while green treefrogs vocalized on vegetation close to the water. Such partitioning suggests that there is minimal physical contact between adults of the two species which may minimize reproductive interference.

#### **IV. OVERALL CONCLUSIONS**

I examined the how the acoustic signals of the invasive Cuban treefrog altered the endocrine physiology of native green treefrogs. Vocal playback experiments indicate that the calls of Cuban treefrog vocalizations do not alter the endocrine physiology of native green treefrogs.

Moreover, I found no evidence that the presence of Cuban treefrogs influences the endocrine physiology of male green treefrogs in natural choruses.

Amphibians are the world's most threatened group of vertebrates with 48% of extant species in decline (Stuart et al., 2004). Invasive species pose one of the greatest threats to this group (Knapp and Matthews, 2000; Kats and Ferrer, 2003). Understanding the different ways these invaders impact native amphibians is crucial to the protection and survival of native species. Outside of the current work, no studies to my knowledge have examined how the courtship signals of invasive species potentially alter the endocrine physiology of native taxa, yet overlap in courtship signals of invasive and native species is well documented. In addition to acoustic signals, olfactory and visual signals produced during courtship are known to modulate hormone levels in conspecific signal receivers (Schubert et al, 2009; Creel et al., 2013). This suggests that, if social signals overlap between invasive and native species, various courtship signals could be altering the endocrine physiology of native taxa. Investigating these signaling dynamics could provide valuable insight into how invasive species negatively impact native taxa.



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## VITA

### Joseph Kennedy

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University of Mississippi  
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#### EDUCATION

Master of Science, Biological Sciences, University of Mississippi, 2019  
Advisor: Dr. Christopher Leary

Bachelor of Science, Biology, Western Carolina University, 2016  
Advisor: Dr. Joseph Pechmann

#### EMPLOYMENT

University of Mississippi 2017 – 2019  
*Teaching Assistant*

Harrison Experimental Forest 2013 – 2016  
*Research Technician*

Western Carolina University 2015  
*Research Technician*

#### RESEARCH EXPERIENCE

Neuroendocrinology Lab, University of Mississippi, Oxford, MS.  
PI: Prof. Christopher Leary, University of Mississippi 2017 – 2019

Herpetology Lab, Western Carolina University, Cullowhee, NC.  
PI: Prof. Joseph Pechmann, Western Carolina University 2012 – 2016

Dusky Gopher Frog Project, Harrison Experimental Forest, Saucier, MS.  
PI: Prof. Joseph Pechmann, Western Carolina University 2013 – 2016

Avian Behavior Lab, Western Carolina University, Cullowhee, NC.  
PI: Prof. Jeremy Hyman, Western Carolina University 2015

## **AWARDS AND FUNDING**

C.H.S. Graduate Student Research in Herpetology (\$1,000) Chicago Herpetological Society	2019
Roger Conant Grants in Herpetology Program (\$500) Society for the Study of Amphibians and Reptiles	2018
Luther Knight Graduate Student Research Grant Opportunity (\$500) University of Mississippi Field Station, Abbeville MS	2018
Luther Knight Graduate Student Research Grant Opportunity (\$500) University of Mississippi Field Station, Abbeville MS	2017
Undergraduate Academic Project Grant (\$220) Honors College at Western Carolina University, Cullowhee NC	2015

## **ACADEMIC APPOINTMENTS**

Comparative Anatomy of Vertebrates: University of Mississippi  
Spring Semester 2017 – 2 lab sections  
Spring Semester 2018 – 2 lab sections  
Spring Semester 2019 – 2 lab sections

Introductory Physiology: University of Mississippi  
Summer Semester 2018 – 1 lab section  
Fall Semester 2018 – 2 lab sections  
Fall Semester 2019 – 2 lab sections

Biological Sciences I: University of Mississippi  
Fall Semester 2017 – 2 lab sections

Inquiry into Life: The Environment: University of Mississippi  
Summer Session 2017 – 1 lab section

## **CONFERENCE PRESENTATIONS**

Southeastern Partners in Amphibian and Reptile Conservation Annual Meeting Black Mountain, NC. Poster – “The role of reproductive interference and endocrine stress in the decline of native green treefrogs following Cuban treefrog invasions.”	2019
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Society of Integrative and Comparative Biology Annual Meeting  
Tampa, FL. Poster – “The role of reproductive interference and endocrine stress in  
the decline of native green treefrogs following Cuban treefrog invasions” 2019

Highlands Plethodontid Conference,  
Highlands, NC. Poster – “Differences in Surface Activity Behaviors in Two  
Terrestrial Salamanders” 2016

Joint Meeting of Ichthyologists and Herpetologists  
New Orleans, LA. Poster – “Differences in Surface Activity Behaviors in Two  
Terrestrial Salamanders” 2016

National Conference on Undergraduate Research  
Asheville, NC. Presentation – “Differences in Surface Activity Behaviors in Two  
Terrestrial Salamanders” 2016

### **EDUCATIONAL OUTREACH**

University of Mississippi Field Station’s Science Day – Abbeville, MS  
Youth Educator – “The Red Spotted Newt” 2018

University of Mississippi Field Station’s Science Day – Abbeville, MS  
Youth Educator – “Identification of Native Frogs” 2017

The Natchez Trace BioBlitz and Wildlife Festival – Tupelo, MS  
Youth Educator – “Native Fauna of Mississippi” 2017