

ANDROGEN MEDIATION OF ELABORATE MALE TRAITS:
THE EFFECTS OF DIHYDROTESTOSTERONE ADMINISTRATION ON VOCAL
QUALITY IN MALE GREEN TREEFROGS, *HYLA CINEREA*

by
Thor Matthew Goodfellow

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2015

Approved by

Advisor: Dr. Christopher Leary

Reader: Dr. Bradley Jones

Reader: Dr. Elaine Day

©2015
Thor Matthew Goodfellow
All Rights Reserved

ACKNOWLEDGEMENTS

I would like to thank Dr. Christopher Leary for his immense help and patience in the preparation of this thesis.

In addition, I would like to thank Sarah Crocker Buta for her aid in the collection of data, the analysis of recorded calls, and the running of statistical tests.

Lastly, I would like to thank my family, friends, and Kathy Lee Barrack who were always there to lift my spirits.

ABSTRACT

THOR MATTHEW GOODFELLOW: Androgen Mediation of Elaborate Male Traits:
The Effects of Dihydrotestosterone Administration on Vocal Quality in Male Green
Treefrogs, *Hyla cinerea*
(Under the direction of Dr. Christopher Leary)

Elevated androgen levels are well known to mediate the expression of male courtship behaviors and elaborate traits, but whether androgens act via threshold or graded effects is less clear. Models linking elaborate male traits to the endocrine system often assume that androgens have a graded effect on the magnitude or extent of elaborate male traits and that female preference for more elaborate male traits thus drives concordant directional selection on circulating androgen levels. To test this hypothesis, I examined the effects of dihydrotestosterone (DHT) administration on the quality of advertisement calls produced by male *Hyla cinerea*. Advertisement calls are an elaborate male trait known to be androgen dependent, and females prefer longer calls produced at a faster rate (i.e., high vocal effort) and calls with carrier frequencies that reflect the mean of the population. Analysis of vocalizations prior to treatment indicated that DHT level was not related to call duration, intercall duration, vocal effort, or call carrier frequency. Analysis of plasma hormone levels from blood samples taken before and after treatment showed that DHT injections elevated androgen levels relative to castor oil-injected controls. However, there was no evidence that androgen administration altered call duration, intercall duration, vocal effort, or carrier frequency of the calls of this species. My results thus provide little support for a graded effect of androgens on vocalization in this species. A graded effect of androgens on male sexual signals is central to current

models explaining the evolution of elaborate male traits and the endocrine system, but modifications to these models may be necessary.

TABLE OF CONTENTS

LIST OF FIGURES VII

LIST OF ABBREVIATIONS VIII

INTRODUCTION..... 1

 MODELS INTEGRATING ANDROGENS AND ELABORATE MALE TRAITS 3

 STUDY SPECIES 7

 ANDROGEN MEASURES 8

METHODS 10

 DATA COLLECTION..... 10

 VOCAL ANALYSIS 11

 COLUMN CHROMATOGRAPHY AND RADIOIMMUNOASSAY PROCEDURE 12

 STATISTICAL ANALYSIS..... 13

RESULTS 14

 RELATIONSHIPS BETWEEN DHT LEVELS AND CALL PARAMETERS PRIOR
 TO TREATMENT 14

 AMBIENT TEMPERATURE PRIOR TO AND SUBSEQUENT TO TREATMENT 14

 HORMONE LEVELS PRIOR TO AND SUBSEQUENT TO TREATMENT..... 20

 VOCAL PARAMETERS PRIOR TO AND SUBSEQUENT TO TREATMENT 20

DISCUSSION 28

REFERENCES..... 32

LIST OF FIGURES

Figure 1. Relationships between hormone concentration and magnitude or extent of trait.....	2
Figure 2. The Energetics-Hormone Vocalization model.....	5
Figure 3. Linear regression showing relationship between circulating DHT level and high carrier frequency of the advertisement call in <i>Hyla cinerea</i>	15
Figure 4. Linear regression showing relationship between circulating DHT level and low carrier frequency of the advertisement call in <i>Hyla cinerea</i>	16
Figure 5. Linear regression showing relationship between circulating DHT level and call duration of the advertisement call in <i>Hyla cinerea</i>	17
Figure 6. Linear regression showing relationship between circulating DHT level and intercall duration of the advertisement call in <i>Hyla cinerea</i>	18
Figure 7. Linear regression showing relationship between circulating DHT level and vocal effort of the advertisement call in <i>Hyla cinerea</i>	19
Figure 8. Circulating DHT, T, and CORT levels prior to and subsequent to treatment with castor oil and DHT.....	21
Figure 9. High carrier frequency of the advertisement call in <i>Hyla cinerea</i> prior to and subsequent to castor oil or DHT treatment.....	22
Figure 10. Low carrier frequency of the advertisement call in <i>Hyla cinerea</i> prior to and subsequent to castor oil or DHT treatment.....	23
Figure 11. Call duration of the advertisement call in <i>Hyla cinerea</i> prior to and subsequent to castor oil or DHT treatment.....	24
Figure 12. Intercall duration of the advertisement call in <i>Hyla cinerea</i> prior to and subsequent to castor oil or DHT treatment.....	26
Figure 13. Vocal effort of the advertisement call in <i>Hyla cinerea</i> prior to and subsequent to castor oil or DHT treatment.....	27

LIST OF ABBREVIATIONS

ICHH	Immunocompetence Handicap Hypothesis
EHV	Energetics-Hormone Vocalization
T	Testosterone
DHT	Dihydrotestosterone
CORT	Corticosterone

INTRODUCTION

Androgens are known to mediate the expression of male courtship behaviors and elaborate traits. In most vertebrate species, for example, seasonal elevation in circulating androgen levels coincides with the onset of mating behavior and the development of secondary sexual characteristics (Nelson 2011). Androgen manipulation studies have repeatedly shown that male courtship behaviors and the development of elaborate male traits, including plumage development and comb growth (in some birds), antler formation (cervid mammals), and vocalization (amphibians, birds, and mammals), are regulated by circulating androgen levels (see citations in Folstad and Karter 1992; Littlejohn et al. 2002; reviewed in Moore et al. 2005). It is not currently well understood, however, how androgen levels relate to the magnitude or extent of male mating behavior or trait elaboration. For instance, androgens may mediate the expression of male traits via threshold effects in which elevated androgen levels result in an increase in mating behavior and trait expression, but elevations above threshold levels do not further alter the extent or magnitude of behavior or trait expression (Figure 1A) (reviewed in Hews and Moore 1997; Leary and Knapp 2014). In contrast, elevated androgens may affect behavior and trait expression in a graded manner. In this case, elevation in androgens above threshold levels result in a concordant increase in the magnitude or extent of behavior or trait expression (Figure 1B) (Hews and Moore 1997; Leary and Knapp 2014).

Threshold and graded effects have become critical in theoretical models aimed at understanding the evolution of elaborate male traits and the endocrine system (Folstad

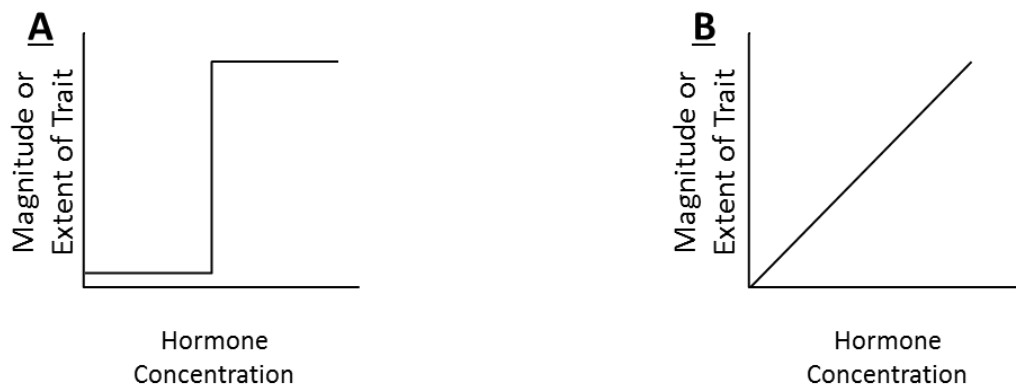


Figure 1. Relationships between hormone concentration and magnitude or extent of trait. A) threshold effect, B) graded or dose-dependent relationship.

and Karter 1992; Emerson 2001). Such models, for instance, propose that graded effects of androgens on male traits preferred by females drive concordant directional selection on circulating androgen levels. The potential negative effects of elevated androgen levels (i.e., immunosuppressive effects), in turn, are predicted to maintain the honesty of sexual signals. The purpose of my honors research was to examine how circulating androgen levels relate to the extent and magnitude of elaborate male traits. Specifically, I assessed how circulating androgen levels mediate variation in vocal parameters known to be important in mate choice in the green treefrog, *Hyla cinerea*.

Models integrating androgens and elaborate male traits

The Immunocompetence Handicap Hypothesis (ICHH, Folstad and Karter 1992) has been particularly influential in directing research aimed at understanding the relationship between circulating androgen level, courtship behavior, and elaborate male traits. The model is rooted in the assumption that androgens have a graded effect on the magnitude or extent of elaborate male traits (Folstad and Karter 1992). For instance, the model predicts that males with higher androgen levels will possess more elaborate sexual traits. However, the ICHH also predicts that elevated androgens weaken the immune system and that such effects prevent some males from having heightened androgen levels and more elaborate male traits (Folstad and Karter 1992). In other words, only males with ‘good genes’, that is genes which confer pathogen resistance, are expected to be able to afford to compromise their immune system in order to increase the magnitude of elaborate male traits (Folstad and Karter 1992). Consequently, the extent or magnitude of elaborate male traits serves as an ‘honest signal’ of genetic quality that is mediated by

circulating androgen levels; mate choice by females is expected to drive directional selection on circulating androgen levels (Folstad and Karter 1992).

The ICHH is thus based upon the central assumption that androgens have a graded effect on the magnitude or extent of elaborate male traits and that this relationship maintains the honesty of sexual signals because of the negative effects of elevated androgen levels on the immune system (Hews and Moore 1997). If the intensity of elaborate male traits fails to accurately track circulating androgen levels (i.e., under a threshold scenario, see Figure 1), an individual could decrease androgen concentration to threshold levels required for full expression of the trait; thus, individuals could effectively avoid any disadvantages caused by heightened circulating androgen levels (e.g., decreased immunocompetence) while maintaining maximum trait expression (Hews and Moore 1997). Whether androgens have a graded or threshold effect on elaborate male traits remains controversial (Hews and Moore 1997; Leary and Knapp 2014). In many instances, for example, androgenic effects on trait expression can be explained by threshold effects (Hews and Moore 1997).

Despite the fact that graded effects of androgens on male sexual traits have been controversial, other more recent models are predicated on the same assumption. For instance, the Energetics-Hormone Vocalization (EHV) model (Emerson 2001), which was intended to describe the variation in vocal quality between calling male anuran amphibians (frogs and toads), proposes that androgens mediate vocal quality in a dose-dependent manner (Figure 2). Specifically, the EHV model predicts that androgen levels increase within and across nights of breeding activity (Figure 2, Emerson 2001) as a result of interactions among competing males (i.e., concepts of the Challenge Hypothesis,

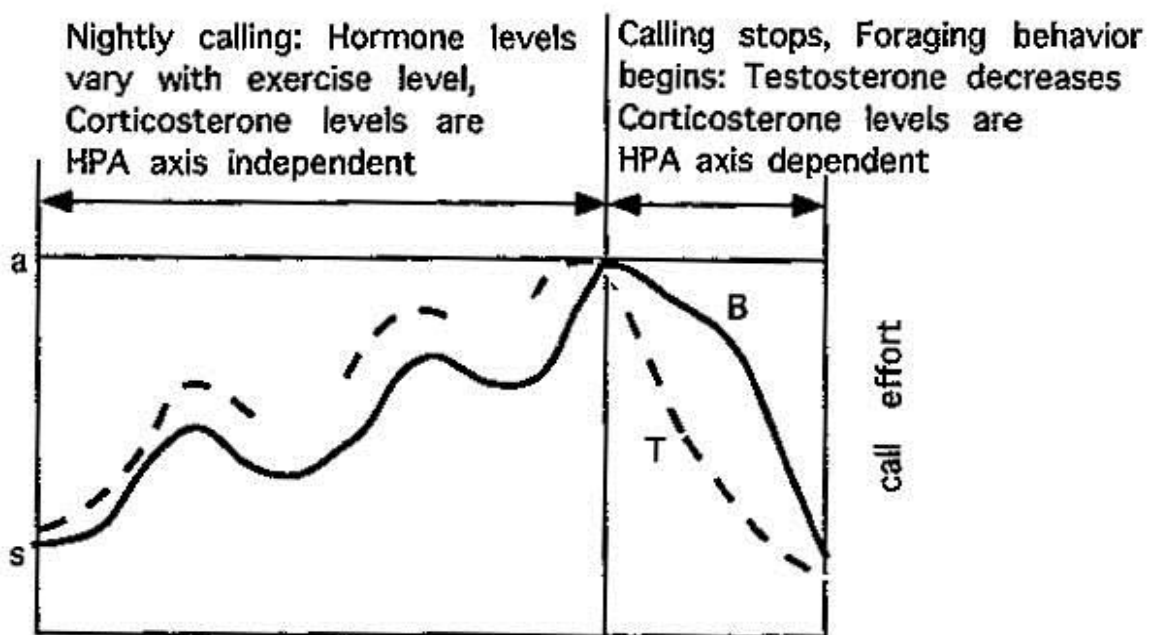


Figure 2. The Energetics-Hormone Vocalization Model (from Emerson 2001). T = testosterone; B = corticosterone; HPA= hypothalamic-pituitary-adrenal axis.

Wingfield 1990). Temporal elevations in circulating androgen levels are predicted to mediate changes in peripheral structures involved in vocalization (i.e., synthesis of myosin in thoracic musculature and an increase in muscle size) that result in an increase in vocal effort (i.e., the energy invested in vocalization) (Emerson 2001). According to the model, as circulating androgen levels rise, individuals expend more energy to produce vocalizations of concordantly greater quality (Emerson 2001). In order to meet the rising energetic demands associated with increased vocal effort, corticosteroid levels are predicted to rise (to promote gluconeogenesis) until levels are reached that down regulate circulating androgen levels and vocal behavior. Thus, the EHV model predicts that temporal variation in androgen levels will be related to variation in vocal quality; males with higher androgen levels are expected to produce more attractive vocal signals (Emerson 2001). Hence, mate selection by females is expected to result in directional selection on circulating androgen levels (Emerson 2001).

While calling behavior in anurans is known to be androgen dependent (reviewed by Moore et al. 2005) evidence for a graded relationship between circulating androgen levels and vocal quality that is central to the EHV model is less clear (reviewed in Leary 2009). Activational effects of androgens on trunk musculature responsible for vocalization are supported in the literature (reviewed in Leary 2009), but whether elevations in androgen levels above threshold levels required to elicit vocal behavior correlate with vocal parameters that are important in mate choice (i.e., vocal parameters related to vocal effort) in a dose-dependent or threshold manner remains controversial. For example, a positive correlation between vocal effort and androgen levels was found across old world tropical frog species (Emerson and Hess 1996); conversely, there is little

evidence that changes in circulating androgens track changes in vocal quality within anuran species (Leary 2009). For instance, while elevated androgen levels were necessary to elicit calling behavior in Banded Wood frogs, *Batrachyla taeniata*, there was no correlation between circulating androgen levels and call duration (Solis and Penna 1997). Furthermore, in Woodhouse's toads, *Bufo woodhousii*, and Great Plains toads, *Bufo cognatus*, no difference in circulating androgen levels was found between calling and non-calling males of the same species (Leary et al. 2004) and there was no evidence that androgen level was related to vocal effort (Leary et al. 2008). Moreover, in American bullfrogs, *Rana catesbeiana*, non-calling males actually have higher androgen levels than calling males (Mendonça et al. 1985).

Study species

I used green treefrogs, *Hyla cinerea*, as a model in which to test predictions of the ICHH (Folstad and Karter 1992) and EHV model (Emerson 2001). Vocal parameters important in mate selection by females of this species include dominant carrier frequency of the call, call duration, and call rate (Gerhardt 1987; Gerhardt 1991). In green treefrogs, dominant calling frequency and call duration are considered static properties that remain relatively constant within an individual; static properties are subject to weak directional or stabilizing selection (Gerhardt 1991). On the other hand, call rate, and thus vocal effort (the energy invested in vocalization per unit time) are dynamic properties that are highly variable within and among individuals; dynamic properties are subject to strong directional selection via mate choice by females (Gerhardt 1991). Specifically, female green tree frogs prefer dominant calling frequencies of approximately 900 Hz (for

low carrier frequency) and 2700 to 3000 Hz (for high carrier frequency), reflecting the mean of the population (Allan and Simmons 1994). In contrast, females of this species (and most other anurans) prefer calls that are longer in duration and produced more frequently (i.e., males with higher vocal effort) (Gerhardt 1987; Gerhardt 1991). Thus, if androgens mediate vocal quality in a graded manner in green treefrogs, as proposed by the ICHH (Folstad and Karter 1992) and the EHV model (Emerson 2001), I expected to find that elevations in circulating androgen levels, above those required to elicit calling behavior, would mediate an increase in call duration and/or call rate (the product of which is vocal effort). Hence, selection for these parameters via mate choice by females should drive concordant directional selection on circulating androgen levels. Because female preference for dominant calling frequency could effectively stabilize selection on circulating androgen levels, I also examined the relationship between androgen level and dominant calling frequency.

Androgen measures

Amphibians have two main androgens: testosterone (T) and dihydrotestosterone (DHT). DHT is better suited than T for exploring androgenic effects on vocal quality. For example, while both DHT and T can directly interact with androgen receptors, T often acts on target tissues after conversion to estrogen via aromatase (Nelson 2011). Conversely, DHT cannot be converted to an estrogen (Nelson 2011). Hence, by manipulating DHT instead of T, direct androgenic effects on vocalization can be ascertained.

I manipulated androgen levels through DHT administration and analyzed vocal parameters known to be important in mate selection by females to examine how androgens mediate vocal quality in male green treefrogs. Threshold versus graded androgenic effects on elaborate male traits are of central importance to both the ICHH (Folstad and Karter 1992) and the EHV model (Emerson 2001) and are thus critical in understanding how the endocrine system potentially mediates variation in sexually selected male traits and the evolution of elaborate males traits. I predicted that DHT administration would result in an increase in vocal parameters important to mate selection. Specifically, I predicted that an increase in DHT levels in calling males would mediate an increase in vocal effort by increasing call duration and/or call rate.

METHODS

Data collection

My study ponds were located at the University of Mississippi Field Station in Lafayette County, Mississippi (34.425, -89.392). *Hyla cinerea* chorus activity was observed on most nights from late May to early July of 2014. Ambient temperatures ranged from 19 °C to 28 °C throughout the breeding period. Observations were typically from 8:00 pm to 12:00 am.

Calling male *H. cinerea* were located and observed using LED headlamps (headlamps had no noticeable effects on behavior). To examine how androgen levels potentially influence vocalization in this species, I first recorded a series of approximately sixty calls from focal males using a Marantz PMD-222 recorder equipped with a Sennheiser ME66 condenser microphone. Animals were then captured by hand, and a blood sample was taken within two minutes via cardiac puncture. Blood samples were placed on ice and returned to the lab where they were centrifuged for 12 minutes at 3000 rpm to separate the plasma which was then stored at -20 °C until radioimmunoassay was carried out. After blood samples were taken, males were weighed to the nearest gram using a portable OHAUS scale, measured to the nearest millimeter from the tip of the snout to the ischium, placed in a cloth bag, and transported to the lab.

At the lab, animals making up the experimental group were injected in the hind limb musculature with 16 µl of DHT in a castor oil vehicle (n=12). Subjects forming the

control group were injected in the hind limb musculature with an equal volume of castor oil only (n=9). All treatments took place on the same night that the animals were collected. Following injections, animals were tattooed on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, U.S.A.). Animals were housed overnight in individual ten-gallon aquaria. Water was provided in the bottom of the aquarium and in a water dish to prevent desiccation, and subjects were fed crickets *ad libitum*.

Subjects were transported back to the study ponds and released back into the chorus approximately 24-48 hours after treatment. The time of return varied due to weather conditions. If males were kept in the lab for longer than twenty-four hours, they were re-injected on the morning of their return to the field. Upon return to the field, subjects were released and observed. If calling behavior was observed, calls were recorded and a second blood sample was taken followed by release of the animal; if calling behavior was not observed, males were left undisturbed and removed from the experiment.

Vocal analysis

Recordings of calls from males were analyzed using Raven Pro 1.4 (Cornell Lab of Ornithology Bioacoustics Research Program, Ithaca, NY, U.S.A.). Spectra, spectrograms, and waveforms were examined for call parameters important to mate choice by females including high and low carrier frequency, duration of call, and intercall duration. Measures were made from 10 consecutive calls and the average values were used to calculate vocal effort using the following equation:

$$Vocal\ Effort = \frac{duration\ of\ call}{duration\ of\ call + intercall\ duration}$$

Column chromatography and radioimmunoassay procedure

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

Plasma samples were analyzed for DHT, T, and CORT in 2 assays. Blood samples taken from a common individual were run in the same assay; otherwise, the samples were randomized between assays.

Statistical analysis

Call parameters, hormone concentrations, and ambient temperatures were entered into StatView (SAS Institute Inc.) and analyzed using linear regression and paired t-tests. Linear regressions were run on pre-injection hormone concentrations to examine the relationship between hormone levels and measured vocal parameters important in mate selection and included pre-injection DHT levels for an additional 10 individuals that were used in a separate experiment to examine the effects of T on calling behavior in this species. A paired t-test was run on pre-injection and post-injection ambient temperatures to test whether differences in vocal parameters were potentially related to differences in temperatures when blood or vocal samples were taken (i.e., body temperature can alter various call parameters in anurans, see Gerhardt and Huber 2002). Paired t-tests were run on pre-injection and post-injection hormone levels of the DHT-injected group and the castor oil-injected group to insure that injections had successfully altered circulating hormone concentrations. Paired t-tests were run on measured vocal parameters for both the DHT-injected group and the castor oil-injected group to examine the effects of the injections on vocalization.

RESULTS

Relationships between DHT level and call parameters prior to treatment

Circulating DHT levels in males prior to injection were examined in relation to vocal parameters known to be important in mate selection by female green treefrogs. Circulating DHT levels were not significantly related to high carrier frequency ($F_{1,29}=4.793 \times 10^{-4}$, $p=0.98$, $r^2=1.653 \times 10^{-5}$) (Figure 3) or low carrier frequency of the advertisement call ($F_{1,29}=0.295$, $p=0.59$, $r^2=0.010$) (Figure 4). Hence, there was no evidence that circulating DHT levels influenced spectral call parameters of the advertisement call that are important in mate choice by females. There was also no evidence that circulating DHT levels were related to temporal characteristics of the advertisement calls. For example, DHT level was not significantly related to call duration ($F_{1,29}=0.264$, $p=0.61$, $r^2=0.009$) (Figure 5), intercall duration ($F_{1,29}=0.122$, $p=0.73$, $r^2=0.004$) (Figure 6), or vocal effort ($F_{1,29}=0.568$, $p=0.46$, $r^2=0.019$) (Figure 7).

Ambient temperature prior to and subsequent to treatment

The ambient temperature of nights when blood and vocal samples were taken was examined to ensure that potential differences in vocal parameters were not due to differences in temperature before and after treatment. There was no evidence of a temperature difference prior to and subsequent to treatment ($t_{20}=1.294$, $p=0.21$).

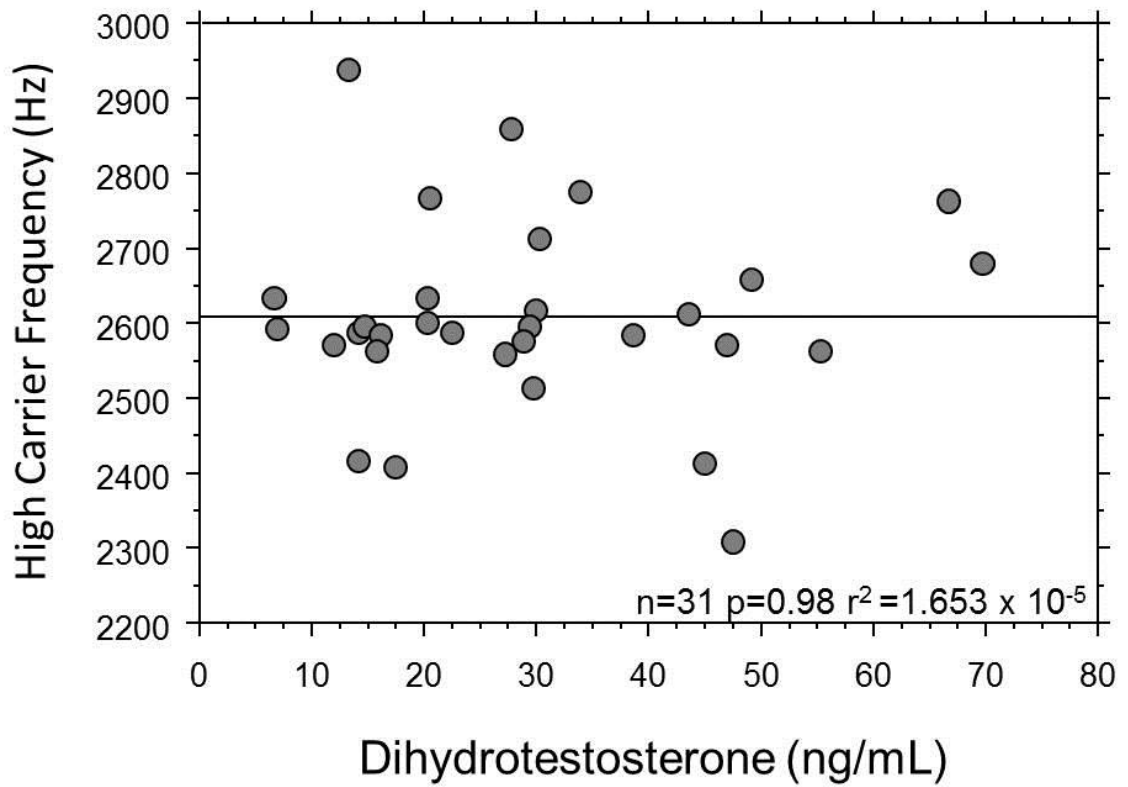


Figure 3. Linear regression showing relationship between circulating DHT level and high carrier frequency of the advertisement call in *Hyla cinerea*.

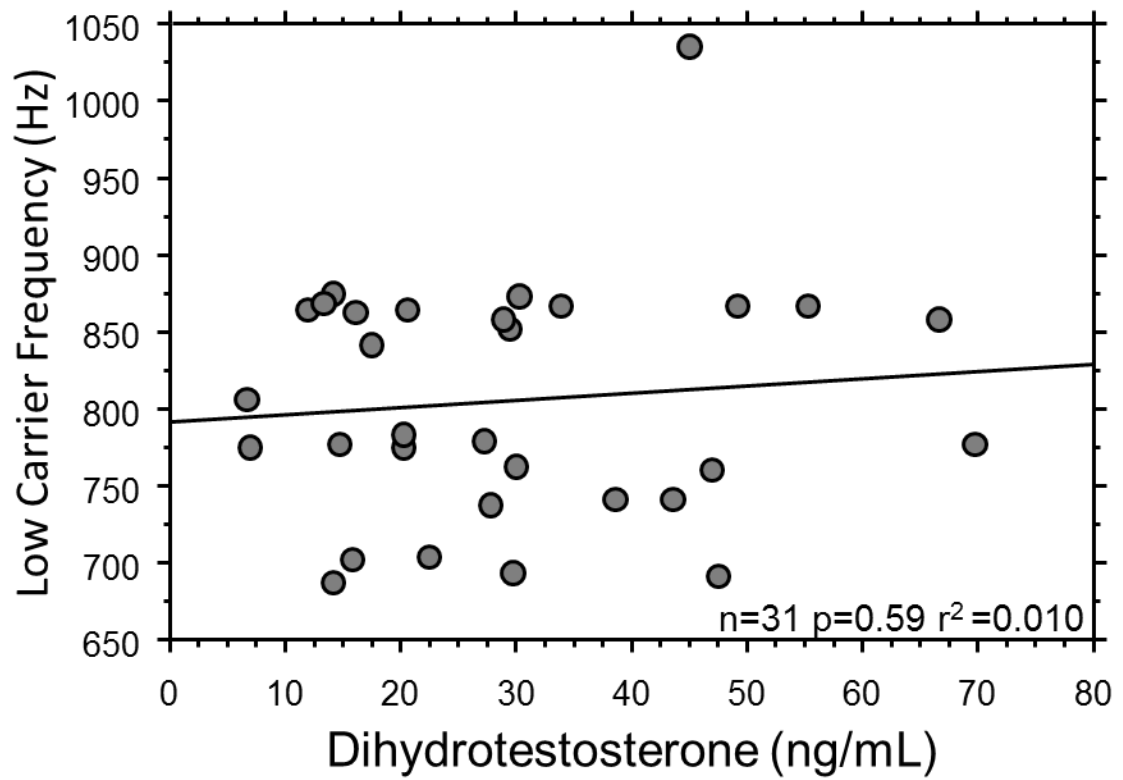


Figure 4. Linear regression showing relationship between circulating DHT level and low carrier frequency of the advertisement call in *Hyla cinerea*.

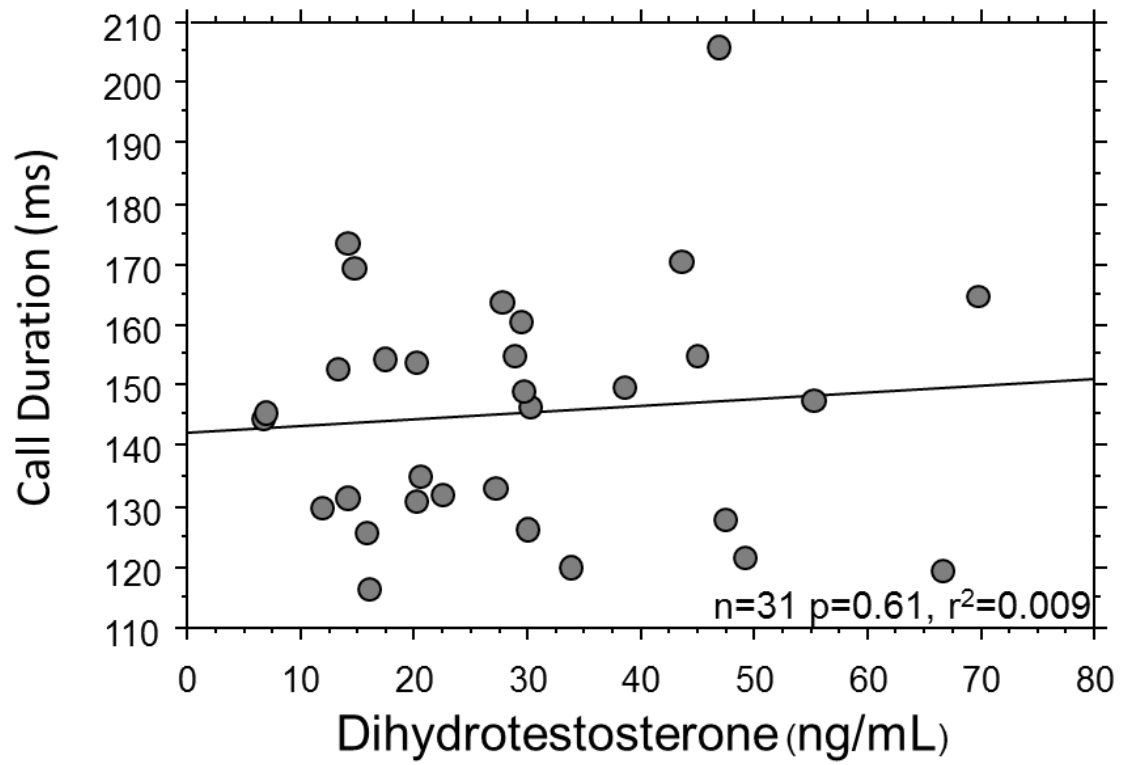


Figure 5. Linear regression showing relationship between circulating DHT level and call duration of the advertisement call in *Hyla cinerea*.

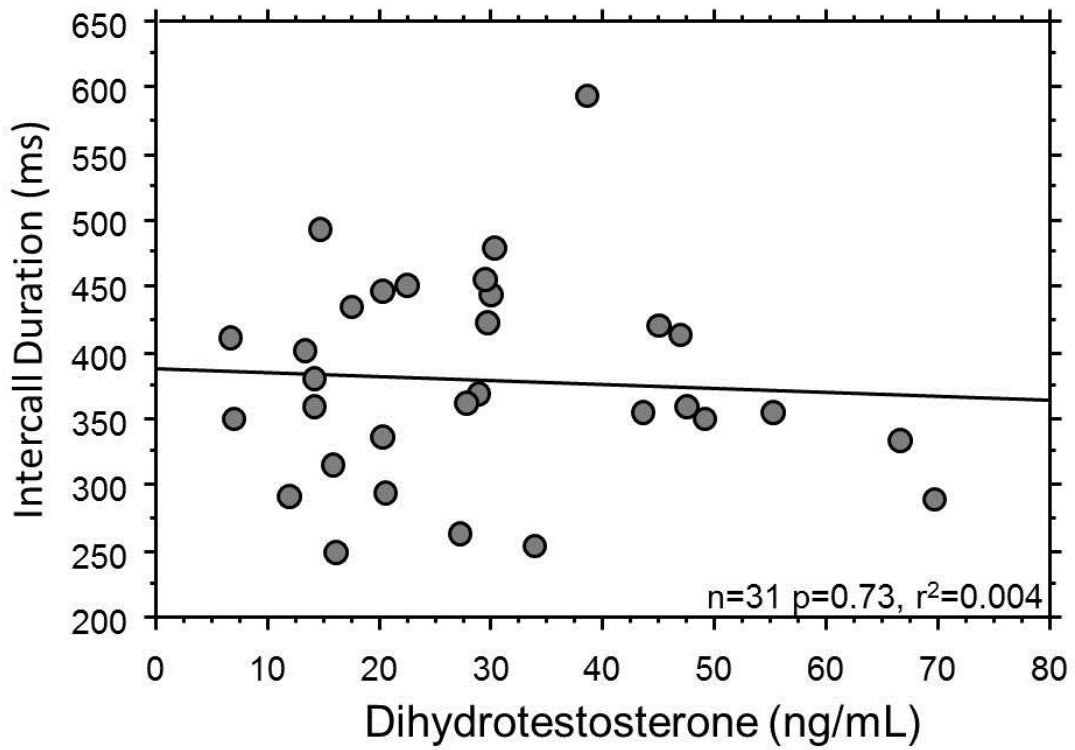


Figure 6. Linear regression showing relationship between circulating DHT level and intercall duration of the advertisement call in *Hyla cinerea*.

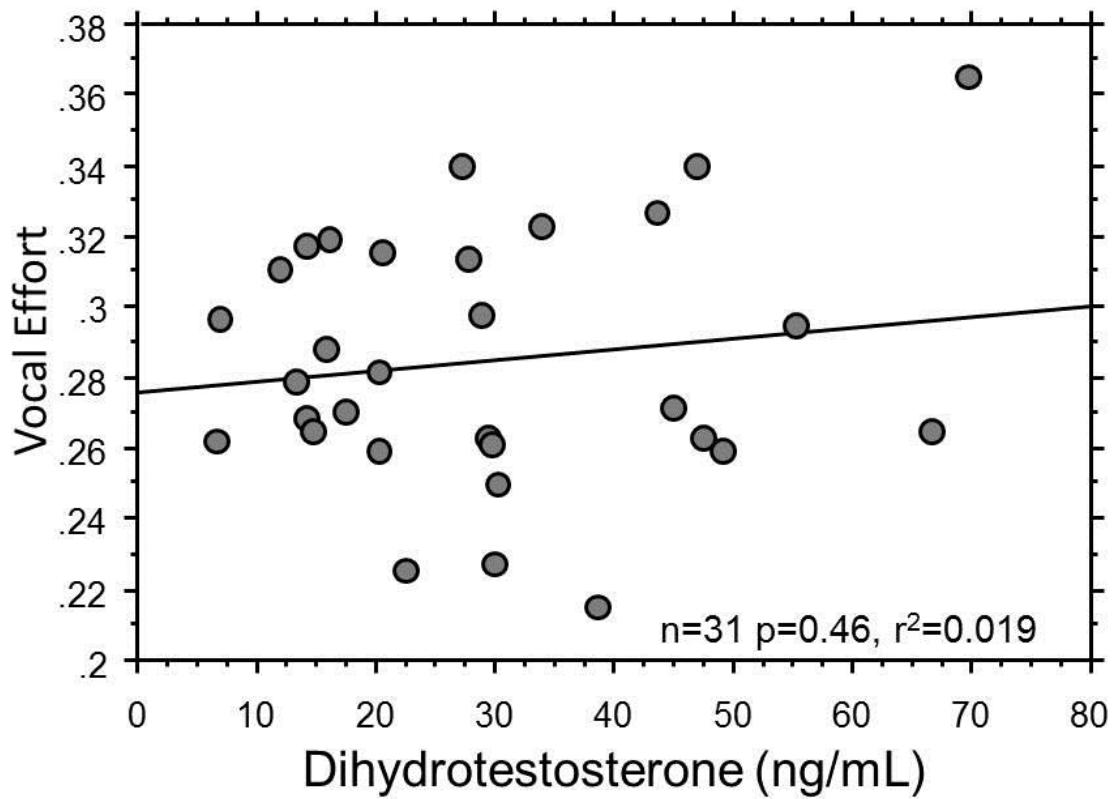


Figure 7. Linear regression showing relationship between circulating DHT level and vocal effort of the advertisement call in *Hyla cinerea*.

Hormone levels prior to and subsequent to treatment

Circulating DHT, testosterone (T), and corticosterone (CORT) levels before and after treatment were examined in the males injected with DHT to ensure that injections successfully elevated DHT. DHT injections resulted in a significant increase in circulating DHT levels ($t_{11}=-3.951$, $p=0.002$) (Figure 8A). DHT injections did not alter circulating T levels ($t_{11}=-0.080$, $p=0.93$) (Figure 8B) or circulating CORT levels ($t_{11}=0.157$, $p=0.87$) (Figure 8C).

Circulating hormone levels before and after treatment were examined in the control group males injected with castor oil to examine the effects of castor oil injection on circulating hormone levels. DHT levels decreased in the castor oil injected control group ($t_8=2.832$, $p=0.02$) (Figure 8A). T levels did not differ prior to and subsequent to castor oil injection ($t_8=1.255$, $p=0.24$) (Figure 8B) nor did circulating CORT levels ($t_8=0.325$, $p=0.75$) (Figure 8C).

Vocal parameters prior to and subsequent to treatment

Vocal parameters known to be important in mate selection by female green treefrogs were analyzed from calls recorded before and after treatment to examine how changes in DHT level altered vocalization. High carrier frequency was not altered in males injected with DHT ($t_{11}=1.386$, $p=0.19$) or males injected with castor oil ($t_8=0.434$, $p=0.67$) (Figure 9). There was a marginal increase in low carrier frequency in males injected with DHT ($t_{11}=-3.325$, $p=0.06$), but this parameter was unchanged in males injected with castor oil ($t_8=0.803$, $p=0.44$) (Figure 10). There was a marginal decrease in call duration in males injected with DHT ($t_{11}=2.068$, $p=0.06$) (Figure 11), but this call

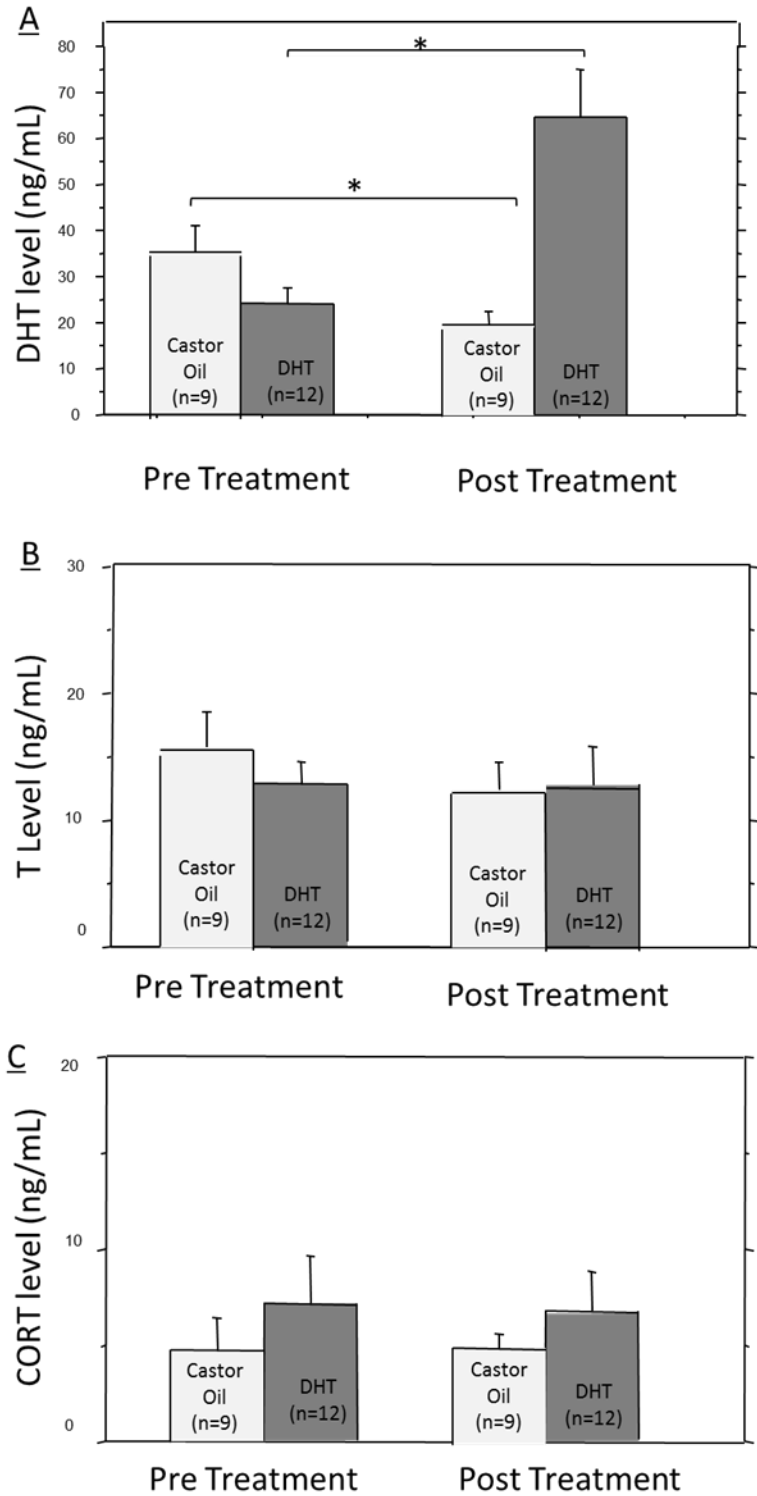


Figure 8. Circulating DHT, T, and CORT levels prior to and subsequent to treatment with castor oil and DHT. Significant differences are indicated with an asterisk.

Whiskers represent standard errors.

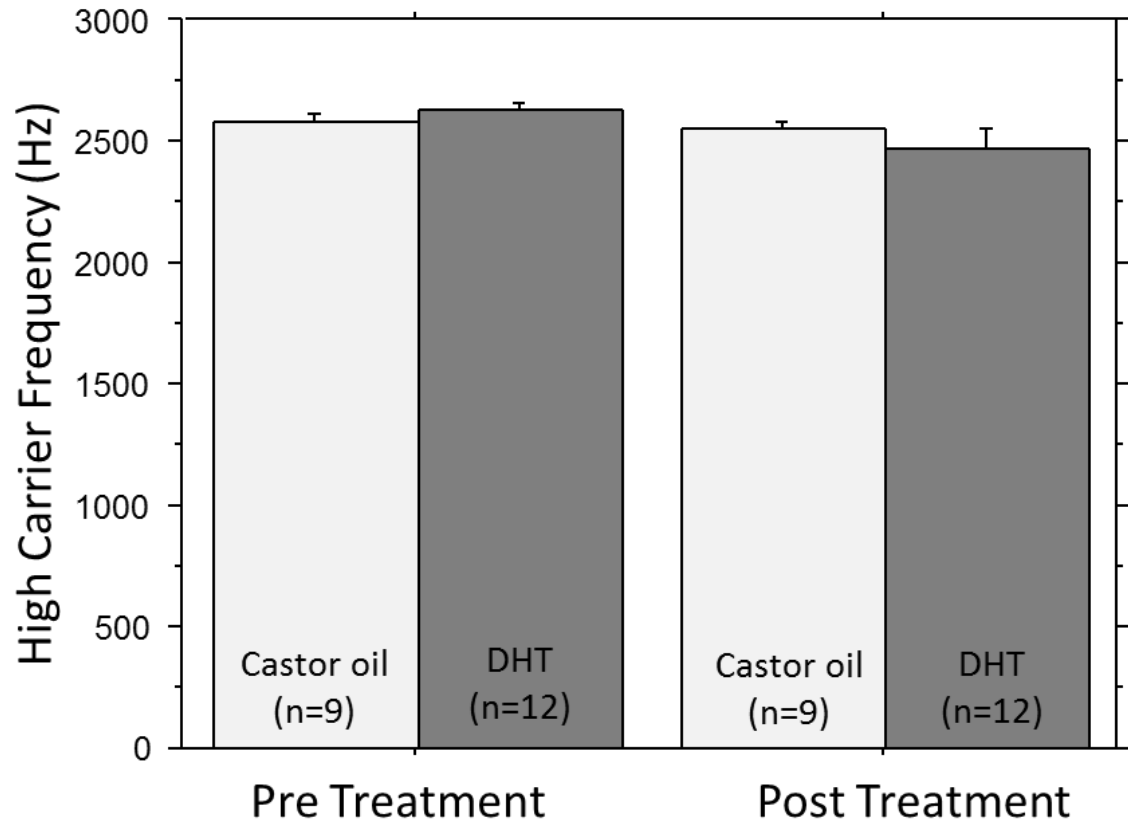


Figure 9. High carrier frequency of the advertisement call in *Hyla cinerea* prior to and subsequent to castor oil or DHT treatment. Whiskers represent standard errors.

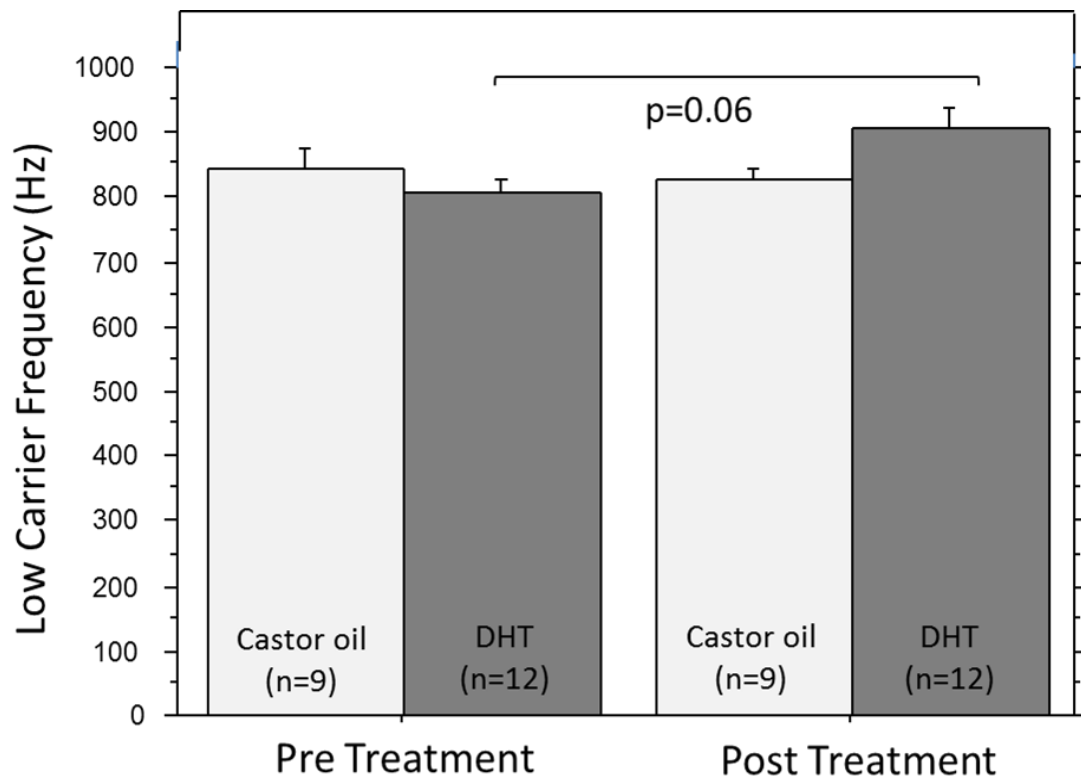


Figure 10. Low carrier frequency of the advertisement call in *Hyla cinerea* prior to and subsequent castor oil or DHT treatment. Whiskers represent standard errors.

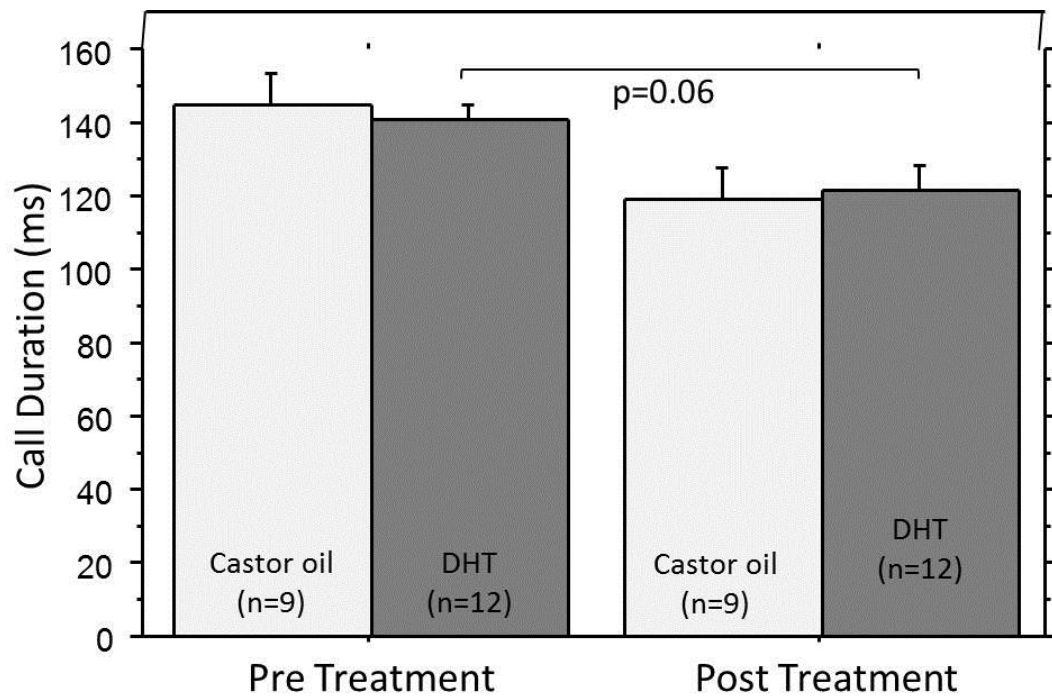


Figure 11. Call duration of the advertisement call in *Hyla cinerea* prior to and subsequent to castor oil or DHT treatment. Whiskers represent standard errors.

parameter was unaltered in males injected with castor oil ($t_8=1.715$, $p=0.12$) (Figure 11). Intercall duration increased in males injected with DHT ($t_{11}=-2.571$, $p=0.02$) and males injected with castor oil ($t_8=-2.467$, $p=0.03$) (Figure 12). Vocal effort decreased in both males injected with DHT ($t_{11}=5.768$, $p=0.001$) and males injected with castor oil ($t_8=3.917$, $p=0.004$) (Figure 13).

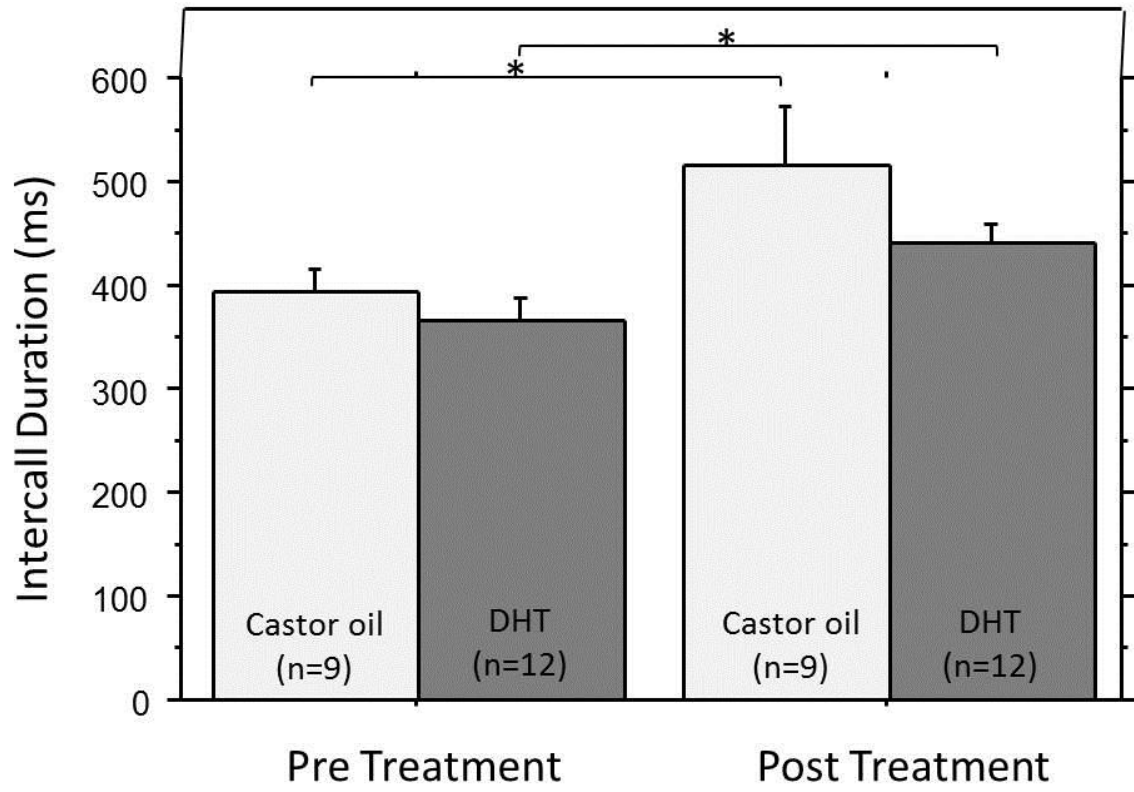


Figure 12. Intercall duration of the advertisement call in *Hyla cinerea* prior to and subsequent to castor oil or DHT treatment. Significant differences are indicated with an asterisk. Whiskers represent standard errors.

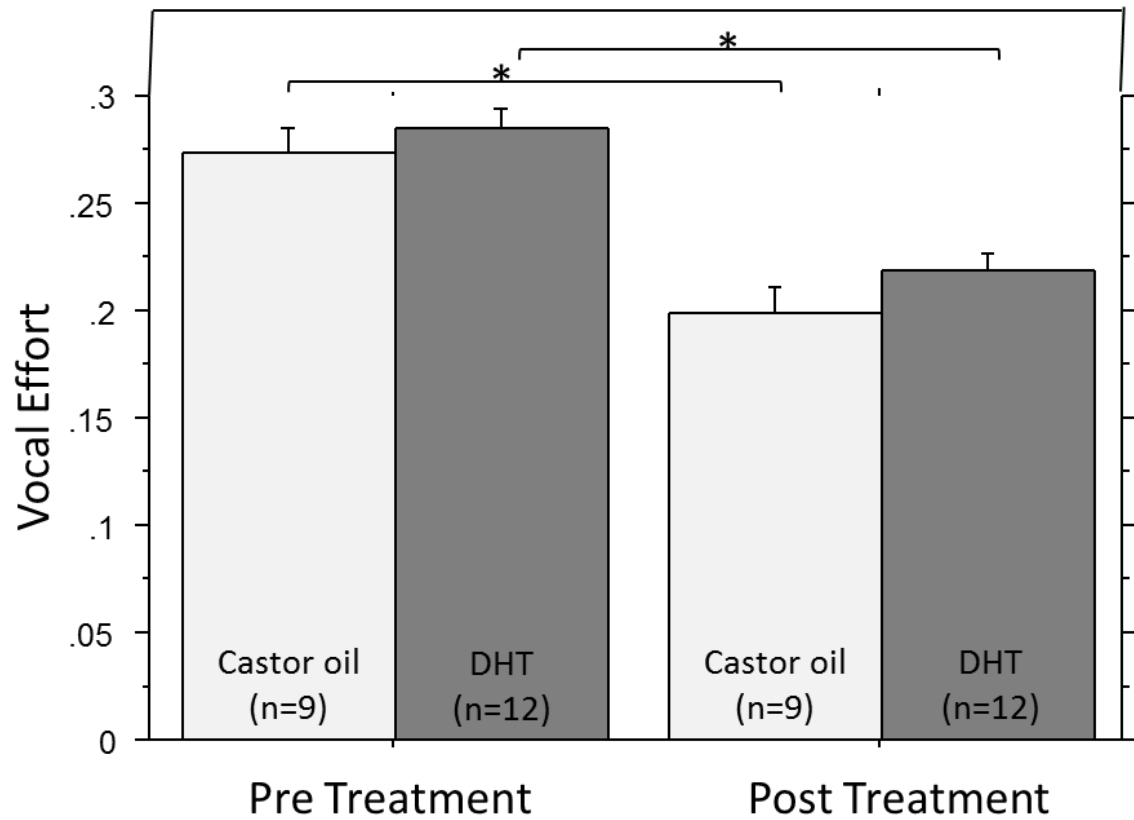


Figure 13. Vocal effort of the advertisement call in *Hyla cinerea* prior to and subsequent to castor oil or DHT treatment. Significant differences are indicated with an asterisk.

Whiskers represent standard errors.

DISCUSSION

My results provide no support for a graded effect of androgens on spectral or temporal features of advertisement calls that are important in mate choice in *H. cinerea*. For example, I found no strong evidence that circulating DHT level in calling male *H. cinerea* was related to call carrier frequency, call duration, intercall duration, or vocal effort. There was also no evidence that DHT injections increased any of the measured vocal parameters.

Injections modified circulating hormone profiles as predicted. The change in plasma DHT levels in the group injected with DHT indicated that injections successfully elevated DHT without altering T or CORT levels. While the decrease in DHT in males injected with castor oil indicates that the injections reduced DHT levels, such a reduction in DHT level is expected. For example, the EHV model (Emerson 2001) predicts that androgens will rise throughout a night of chorus activity in response to male-male interactions (i.e., concepts of the Challenge Hypothesis, Wingfield 1990) and decrease in the absence of interactions with other males. DHT probably decreased in control males because of the lack of male-male interactions while held in the lab. The reduction in DHT levels in controls undoubtedly contributed to significant differences in circulating DHT levels in castor oil-injected and DHT-treated subjects. Nonetheless, even with this dramatic difference in circulating DHT levels between treatment groups, there was no evidence of a clear difference in any of the measured vocal parameters for castor oil and

DHT-treated males. Thus, there was no evidence that DHT mediated changes in vocal quality.

My results do not support predictions of the ICHH (Folstad and Karter 1992) or the EHV model (Emerson 2001), which both predict a positive dose-dependent relationship between androgen levels and the magnitude or extent of sexual signals or elaborate male traits. Such models have been highly influential in directing research aimed at understanding variation in sexually selected traits among males and the evolution of elaborate male traits and the endocrine system (Leary and Knapp 2014; Hews and Moore 1997). For example, the ICHH (Folstad and Karter 1992) predicts that elaborate male traits serve as an ‘honest signal’ of ‘good genes’ via positive effects of androgens on trait elaboration and negative effects on the immune system; thus, only males with genes conferring pathogenic resistance can afford to express more elaborate traits. Similarly, the EHV model (Emerson 2001) describes variation in vocal quality of male anurans in the context of graded effects of androgens on vocal parameters. Hence, both the ICHH (Folstad and Karter 1992) and the EHV model (Emerson 2001) predict that female preferences for more elaborate male traits will drive directional selection on circulating androgen levels. While these evolutionary models rely on graded androgen mediation of elaborate male traits, most research cited to support this relationship can be explained by a threshold effect; that is, elevated androgen levels appear to cause an increase in trait expression up to a threshold hormone level after which further elevation in androgen concentration does not alter the trait (Hews and Moore 1997). Likewise, my results suggest that a threshold relationship is likely to exist between androgen level and vocalization in *H. cinerea*. Previous work has shown that elevated androgens are

necessary to elicit vocal behavior in anuran amphibians (Penna et al. 1992, Moore et al. 2005). My results suggest that elevations in androgen levels, above that required to elicit the behavior, does not improve the quality of the calls. Because the extent of elaborate male traits does not accurately reflect circulating androgen levels, males would be expected to down regulate androgen levels to threshold levels to avoid the potential costs of elevated androgens, such as a weakened immune system (Folstad and Karter 1992). Furthermore, selection by females for more elaborate male traits is unlikely to drive directional selection on circulating androgen levels.

As graded androgen mediation of elaborate male traits is often not supported, the ICHH (Folstad and Karter 1992) and the EHV model (Emerson 2001) should be modified if they are to continue to direct research on the evolution of elaborate male traits and the endocrine system. An interesting modification involves the integration of glucocorticoids with androgen-based models of sexual selection (Leary and Knapp 2014). For example, one proposed model suggests that, androgens and glucocorticoids work in concert to mediate elaborate male traits; under this model, low to medium levels of circulating glucocorticoids elevate the extent to which a trait is expressed whereas high levels of circulating glucocorticoids down regulate the magnitude of the trait (Leary and Knapp 2014). This results in selection for elaborate male traits via mate choice driving selection on glucocorticoid levels and androgen levels (Leary and Knapp 2014). While Emerson (2001) includes a positive relationship between glucocorticoids and elaborate male traits in her model, this relationship is predicted to be a byproduct of the increased energy demands associated with androgenic effects on vocal effort. Increased glucocorticoids, for instance, were expected to promote the mobilization of energy reserves associated

with increased vocal effort. If androgens and glucocorticoids mediate elaborate male traits together, an elevation in circulating levels of both DHT and glucocorticoids may be necessary to increase vocal effort. Future studies could address this problem by injecting both DHT and CORT to examine the effects on vocalization.

Another possibility for the lack of DHT-mediated effects on vocalization in *H. cinerea* is that androgens actually act through estrogen metabolites to influence calling behavior (Nelson 2011). Because DHT was the androgen administered in this experiment and is not converted to estrogens, only effects from androgen receptor complexes could be studied, leaving the possibility that androgen mediation of elaborate male traits is a result of T aromatization to estradiol in target tissues (Nelson 2011). A concurrent Sally McDonnell Barksdale Honors College thesis is examining this possibility.

In summary, my research suggests that androgens are more likely to mediate calling behavior in *H. cinerea* via threshold effects rather than graded effects. However, models currently used to explain the evolution of secondary sex characteristics and the endocrine system rely on a graded relationship. We need to begin considering new models that incorporate threshold effects of androgens on elaborate male traits to better understand how hormones potentially affect the evolution of elaborate male traits and how the evolution of elaborate male traits potentially affects the evolution of the endocrine system.

REFERENCES

- Allan, SE & Simmons, AM 1994, 'Temporal features mediating call recognition in the green treefrog, *Hyla cinerea*: amplitude modulation', *Animal Behavior*, 47, 5, pp. 1073-1086.
- Emerson, S, & Hess, D 1996, 'The role of androgens in opportunistic breeding, tropical frogs', *General And Comparative Endocrinology*, 103, 2, pp. 220-230.
- Emerson, SB 2001, 'Male advertisement calls: behavioral variation and physiological processes', In: *Anuran Communication*, Ed. M.J. Ryan, Washington, DC, Smithsonian Institution, pp. 36-44.
- Folstad, I, & Karter, A 1992, 'Parasites, Bright Males, and the Immunocompetence Handicap', *The American Naturalist*, 139, 3, pp. 603-622.
- Gerhardt H, & Huber F 2002, *Acoustic communication in insects and anurans*, Chicago, University of Chicago Press.
- Gerhardt, H 1987, 'Evolutionary and neurobiological implications of selective phonotaxis in the green treefrog, *Hyla cinerea*', *Animal Behavior*, 35, 5, pp. 1479-1489.
- Gerhardt, H 1991, 'Female mate choice in treefrogs: static and dynamic acoustic criteria', *Animal Behavior*, 42, 4, pp. 615-635
- Hews, DK & Moore, MC 1997, 'Hormones and sex-specific traits: critical questions'. In: *Parasites and pathogens: Effects on host hormones and behavior*, Ed. N.E. Beckage, New York, NY, Chapman & Hall, pp. 277-292.

- Leary, C, Garcia, A, Knapp, R, & Hawkins, D 2008, 'Relationships among steroid hormone levels, vocal effort and body condition in an explosive-breeding toad', *Animal Behaviour*, 76, pp. 175-185.
- Leary, C, Jessop, T, Garcia, A, & Knapp, R 2004, 'Steroid hormone profiles and relative body condition of calling and satellite toads: Implications for proximate regulation of behavior in anurans', *Behavioral Ecology*, 15, 2, pp. 313-320.
- Leary, CJ 2009, 'Hormones and Acoustic Communication in Anuran Amphibians', *Integrative and Comparative Biology*, 49, pp. 452-470.
- Leary, CJ, & Harris, S 2013, 'Steroid hormone levels in calling males and males practicing alternative non-calling mating tactics in the green treefrog, *Hyla cinerea*', *Hormones And Behavior*, 63, pp. 20-24.
- Leary, CJ, & Knapp, R 2014, 'The stress of elaborate male traits: integrating glucocorticoids with androgen-based models of sexual selection', *Animal Behaviour*, 89, pp. 85-92.
- Mendonça, M, Licht, P, Ryan, M, & Barnes, R 1985, 'Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels', *General And Comparative Endocrinology*, 58, 2, pp. 270-279.
- Moore, F, Boyd, S, & Kelley, D 2005, 'Historical perspective: Hormonal regulation of behaviors in amphibians', *Hormones And Behavior*, 48, pp. 373-383.
- Penna, M, Capranica, R, & Somers, J 1992, 'Hormone-induced vocal behavior and midbrain auditory sensitivity in the green treefrog, *Hyla cinerea*', *Journal Of*

Comparative Physiology. A, Sensory, Neural, And Behavioral Physiology, 170, 1, pp. 73-82.

Solis, R, & Penna, M 1997, 'Testosterone levels and evoked vocal responses in a natural population of the frog *Batrachyla taeniata*', *Hormones And Behavior*, 31, 2, pp. 101-109.

Wingfield, JC, Hegner, RE, Dufty, AM, & Ball, GF 1990, 'The 'Challenge Hypothesis': Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies', *The American Naturalist*, 136, 6, pp. 829-846.