COORDINATED COMMUNICATION: AN ANALYSIS OF SIGNAL AND PREFERENCE PHENOTYPES IN THE GENUS HYLA

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The undersigned, appointed by the dean of the Graduate School,

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COORDINATED COMMUNICATION: AN ANALYSIS OF SIGNAL AND PREFERENCE PHENOTYPES IN THE GENUS *HYLA*

Presented by Jessica Ann Merricks

A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

Professor H. Carl Gerhardt

Professor James A. Birchler

Professor Todd R. Schachtman

Professor Johannes Schul

This work is dedicated to my family, for all their love and support.

Thank you for believing in me, even on the days when I did not believe in myself.

Now we can celebrate because, yes, I am finally done with school.

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ABSTRACT

Pre-mating isolation is often accomplished through the use of communication signals. Generally, species differ in their mate-attracting signal and conspecific receivers show strong discrimination based on these signal features. This coordination aids in the prevention of potentially costly hybridization because individuals that produce signals with values that differ greatly from the population mean are unlikely to attract a mate, and receivers with extreme preferences may not find a mate producing the preferred signal values. Selective pressures shape both male and female reproductive behaviors, often mediating the degree of variation they contain and ultimately resulting in a tight association between the two. Over the past several decades, scientists have argued about how a tightly matched communication system can be maintained between sender and receiver. The first goal of my dissertation was to investigate how unlearned signal and preference phenotypes are inherited from one generation to the next. Using the F_1 hybrids of two closely related treefrogs species, I quantified the behavioral phenotypes of male and female siblings in order to determine if the genetically determined male acoustic signal and female preference criteria aligned with each other, or if the coupling of these behaviors would be disrupted due to hybridization. I investigated the behaviors of hybrids between the sister species, *Hyla chrysoscelis* and *H. versicolor*. Males of both species broadcast a simple, rhythmic signal which follows a distinct temporal pattern. Females generally prefer the species-specific call properties, with minimal variation. The calls and preferences of these two species differ in several important ways, providing an opportunity to track both

behaviors in F_1 individuals. After raising the hybrids to sexual maturity, I recorded and analyzed the advertisement calls of males and subjected females to preference tests in which they were given a choice between a representative call of either parental species and the call of hybrid males. My results clearly do not provide evidence of genetic coupling in this system. While I would have expected to see intermediate phenotypes in both males in females simply to do additive effects in the F_1 generation, I saw clear evidence of dominance from one parent, depending on the certain traits. Future behavioral work (beyond the F_1) as well as genetic analysis is needed to further determine the path of inheritance of signals and preferences in closely related hylids.

Despite the tight coordination of signals and preferences within species, variability of signal traits can occur among and within individuals. Variation is often seen in the presence of changing conditions, such as the level of competition, which can drive individuals to implement signaling strategies to increase their ability to obtain mates. These often involve adjusting the frequency, duration, or intensity of those signals, or switching to distinctive aggressive signals, in order to increase their receptivity to females or repel their competitors. My second goal was to learn more about the role of signal plasticity as it relates to male competition and mate choice. To study the role of social context on signal variability, I analyzed the behavior of males and females of the pine woods treefrog, *Hyla femoralis,* which broadcasts a highly variable signal and calls from densely populated breeding aggregations. I investigated the extent of behavioral variability in this species in the context of competition, and also how this signal plasticity influences mate choice. My behavioral results indicate that males are strongly influenced by

competitor behavior, and that individuals modify their signals on a fine-temporal scale. Females showed strong preferences to fast, static rates, a behavior which is not commonly displayed by conspecifics for extended periods, indicating that selective pressure from females is probably weak for most acoustic signals in this species. Females also surprisingly show preference for the males in the following position when competitors overlapped, suggesting that physical masking may be an important perceptual process driving mate choice. This work speaks to the relative strength of female preferences for signal timing, specific signal properties and their interaction in an effort to enhance our understanding of signal plasticity in the dynamic social context of breeding aggregations.

CHAPTER 1

INTRODUCTION

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The amazing degree of diversity on earth is due to population divergence and ultimately, speciation. Reproductive isolation between populations is crucial for speciation to occur, and much attention has been given to the factors that lead to such isolation (Coyne and Orr, 2004). Behavioral isolation has been cited numerous times as a strong driver of reproductive isolation between species (Gleason et al., 2002**;** Hoikkala et al. 2000). While natural selection and genetic drift certainly drive population divergence, sexual selection can also lead to divergence by acting on variance in mating success (Darwin, 1871; Lande, 1981; West-Eberhard, 1983). Since Darwin's time, researchers have focused their attention on the variation in heritable traits and the degree to which both natural and sexual selection act upon them. Sexual selection receives special attention because both mate choice and competition can act to shift the degree of variation in both sexes. Generally, reproductively isolated species differ in some aspect of behaviors which aids in the prevention of potentially costly hybridization (Dobzhansky, 1970). Selective pressures shape genotypic expression in both sexes, often mediating the degree of variability in the communication system and leading to a tight coordination between phenotypes.

Understanding the underlying genetic architecture of reproductive behaviors is important for informing the process of speciation (Coyne and Orr 1989, 2004). Behaviors under polygenic control are most often cited as important for causing reproductive isolation (Mackay et al., 2005), but there is tremendous contrast in the studies on genetic divergence, with some claiming many loci of small effect (Type I genetic architecture) and yet other citing few loci of large effect (Type II genetic architecture) (See Arbuthnott, 2009

review, Templeton, 1981; Falconer et al., 1996). Some authors have argued that for those species in which behaviors are controlled by fewer loci, speciation is more likely to occur rapidly because the reproductive isolating behaviors are tightly linked, causing selection on both traits simultaneously (Arbuthnott 2009; Kronforst et al., 2006). Most of the work on the genetic architecture of behavioral traits have focused on insects because of the ease with which multiple generations can be raised. In addition, insect courtship is typically dictated by behaviors which are simple and easily quantifiable. Studies of Hawaiian crickets and *Heliconius* butterflies provide some of the best genetic evidence for a coupling of genes controlling the expression of sexual signals and mate preferences via linkage disequilibrium. Other studies have provided robust evidence against the genetic coordination of these traits by this mechanism (Ritchie, 2000; Rosenthal et al., 2003). Understanding the underlying genetic basis of these traits is important because these patterns also relate to the evolutionary forces driving variation of quantitative traits (Mackay, 2004).

In addition to understanding the genetic mechanism driving reproductive behavior, it is important to understand that the degree of variation in certain sexual traits is an evolutionary consequence of selection on those traits. One common measure of selection on sexual traits is mating preferences. While measuring female preferences do not always translate directly to mating success, researchers have documented its importance in the evolution of secondary sex traits studying across a wide range of taxa (Andersson, 1994; Thornhill and Alcock, 1983). This is especially true of species that reproduce in leks and other breeding aggregations. In leks, males gather and display in a specific area to attract

and secure mates (Alatalo et al., 1996). Given this scenario with large numbers of individuals gathered for the purpose of reproduction leks and other large breeding aggregations have been the focus of several empirical and theoretical studies involving sexual selection via mate choice and male competition (Andersson 1994; Kirkpatrick and Ryan, 1991), as both have the potential to exert major changes in the direction and degree of variation in populations. The reason is that relatively few males may be highly successful at mating and hence the behaviors that mediate this success will be strongly favored by selection. The highly competitive nature of leks requires males to employ strategies to increase their receptivity to their intended receiver (Martinez-Rivera and Gerhardt, 2008; Klump and Gerhardt, 1987; Fischer et al., 2002; Lim and Greenfield, 2007; Zelick and Narins, 1985; Brumm, 2006), which also makes them an excellent model for studying the forces of sexual selection. Complex breeding aggregations often involve multiple species. Here females face the problems of avoiding mating with heterospecific males as well as choosing among conspecifics. Furthermore, they must contend with considerable background noise created by large numbers of individuals. Such social contexts highlight the importance of understanding the mechanism underlying behavioral isolation.

Anurans (frogs and toads) commonly form breeding aggregations in and around bodies of water. Females must lay their eggs in water, and hence the lek location is a resource to which they must come. Here, males from one or more species gather in large aggregations to signal their presence to females. In a few species, such as green and bullfrogs, males defend specific parts of a pond that contain particularly favorable egg-laying sites, but in most species that form huge choruses, females select a mate and carry

him on their backs to an oviposition site of their choosing. Breeding choruses have been a common focus of animal behavior research for several decades (Gerhardt and Huber, 2002).

The acoustic signals of males that do not defend calling sites are generally stereotyped, allowing researchers to easily classify species-specific characteristics that might be under selective pressure. Both the temporal and spectral aspects of advertisement calls are well documented in the literature (see review in Gerhardt, 1994). The acoustic signals are recorded easily in field and laboratory settings. Female preferences are readily quantified using well-established phonotaxis experiments in the lab as well as in the field (see Wagner, 1998; Gerhardt, 1982; Grafe, 1999; and Schwartz et al., 2001). From these and other experiments, it is clear that females possess specific recognition mechanism to identify and orient towards appropriate mates (Gerhardt, 1982; Bush et al., 2002; Gerhardt and Huber, 2002). For most holoarctic hylids, fine-temporal patterns are typically important for mate recognition. Stereotyped temporal patterns are useful for localization of conspecifics at close range (and lower densities), and females can often discriminate between two males based on temporal pattern (Grafe, 1999; Gerhardt et al., 2000; Bush et al., 2002).

Since both signals and preferences are unlearned in anurans, they offer an opportunity to track the genetic inheritance of these traits and potentially provide explanations for the coordination of signals. In **Chapter 2**, I investigated the coordination of signal and preference phenotypes in closely related treefrog species through a study of their F_1 hybrids. My goal was to determine if these behavioral phenotypes, which are

tightly linked within each species, would be disrupted in first generation hybrids. I used the distribution of male signal behaviors as well as preference data to speculate on potentially genetic mechanisms underlying the inheritance of these behaviorally isolating traits within each species.

Despite the tight coordination of signals and preferences within species, variability of signal traits can occur among and within individuals. For the rest of my dissertation, I wanted to learn more about the role of signal plasticity as it relates to male competition and mate choice. Despite a noisy interspecific breeding pond, females are able to discriminate against unsuitable heterospecific mates (Schwartz and Gerhardt, 1989). Males in anuran choruses also implement a variety of signal modifications in an effort to increase their mating success. Many species produce a distinct aggressive signal, which is used to repel rivals from calling sites (Fellers, 1979; Wagner Jr, 1989; Bee, 2003; Reichert, 2011). Others modify the timing of their signal in order to secure an optimum calling position relative to close neighbors (Schwartz and Wells, 1985; Martinez-Rivera and Gerhardt, 2008).

Much attention has been given to understanding selective forces underlying species-specific signal traits (Searcy and Andersson, 1986; Slabbekoorn and Smith, 2002); (Gerhardt, 1991). Many other studies have focused on signaling strategies used to outcompete rivals (Fischer et al, 2002; Lim and Greenfield, 2007; Zelick and Narins, 1985, Schwartz, 1994). Unfortunately, few studies have looked at the interaction between these two important behavioral characteristics. While females may show strong selection for specific signal features, often those features are distorted in a noisy chorus environment

(Bee, 2008; Wollerman and Wiley, 2002; Wollerman, 1999). In addition, some signaling strategies involve switching to different types of signals (e.g. aggressive calls), which in some cases detract from attracting females (Grafe, 1995; Marshall et al., 2003).

With regard to adjustments in signal timing, neighboring signalers may adjust the timing of calls relative to one another, resulting in perfect synchrony, or the signals may fall 180 degrees out of phase, resulting in perfect alternation (Greenfield, 1994; Hartbauer et al., 2012). Between these two extremes are circumstances in which individuals may adjust signal timing in order to avoid overlapping with neighbors or to secure the leading position in a series of signal broadcasts (Martinez-Rivera and Gerhardt 2008; Wells and Schwartz, 1984; Grafe, 1999). The preference criteria of females may drive these behaviors if females are selective for signal timing between neighbors (Dyson and Passmore, 1988; Bosch and Márquez, 2002).

A few other studies in insects and anurans have looked at the relative importance of signal features and signal timing. Many have focused on differences in signal amplitude and frequency (Snedden and Greenfield, 1998; Römer et al., 2002; Klump and Gerhardt, 1992); Grafe, 1996). Höbel (2010) investigated the interaction between signal timing and temporal features in the advertisement calls of the green treefrog, *Hyla cinerea.* She found that female preferences for certain signal traits varied in strength relative to signal timing, suggesting that overlap does not necessarily prevent females from orienting to the source of a preferred signal (Höbel, 2010). It is clear that the social context may play an important role regarding which signal traits are most important to females, and potentially subjected to stronger selection.

The pine woods treefrog, *Hyla* femoralis, presents in interesting case upon which to investigate questions regarding signal variation, plasticity, and female preferences. Among North American frogs, and especially the hylids, fine-temporal traits such as the pulse-repetition rate (pulse rate) usually show little within-individual variability (Gerhardt, 1991). *H. femoralis* is unique in that this property of their advertisement signals is highly plastic (Gerhardt, 1974; Gerhardt and Huber, 2002; personal observation). Females of this species face additional challenges due to the atypical nature of conspecific calls, in which pulses are produced at irregular intervals for many minutes rather than being organized into discrete trains (or calls) as in all other North American species in the genus *Hyla*. It is unclear if females base mating preferences on fine-temporal patterns, signal timing interactions between neighbors, or both.

In **Chapter 3**, my goal was to understand the role of dynamic signal traits in mate choice and competition, and how within-male variability plays a role in the evolution of signals in general. I quantified the degree of variation in the male advertisement call under different levels of competition (measured by chorus density). I also designed experiments to measure fine-temporal adjustments by males in response to nearby neighbors in order to examine if males actively modulate features of the signal or the relative timing in response to calling neighbors.

Finally, in **Chapter 4**, I investigated female preferences in *H. femoralis*, in an effort to understand the relative strength of selection on both signal features and timing interactions. My results address a significant gap in our understanding about signal plasticity as it relates to female preferences and speaks to the relative strength of female

preferences for signal timing as well as identifying specific signal properties that could be used to reliably identify conspecific males. My goal was to enhance our understanding of signal plasticity in the dynamic social context of breeding aggregations by experimental studies of a species in which call variability is paramount.

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CHAPTER 2

BEHAVIORAL ANALYSIS OF SIGNALS AND PREFERENCES OF INTERSPECIFIC HYBRID TREEFROGS

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INTRODUCTION

For closely related sympatric species, behavioral isolation is a consequence of the fact that the mate-attracting signals of male calls are species-specific and females prefer the signals of conspecific males. The match between signal properties and female preferences can arise and be maintained simply from mutual selection by the two sexes. Males that produce signals with values that differ greatly from the population mean are unlikely to attract a mate, and females with extreme preferences may not find a male producing calls with acceptable values. The question that then arises is: how can signals and preferences diverge in a parallel fashion during speciation and ultimately achieve the behavioral isolation observed in assemblages of different species breeding at the same time and place? One controversial hypothesis, genetic coupling, postulates that species-specific signal properties and receiver selectivity for those properties are under common genetic control (Hoy et al., 1977; Greenfield, 2002; Hoy and Paul, 1973). Such a mechanism could promote rapid divergence because mutations affecting either signals or preferences would still result in an instant match between senders and receivers.

Over the past several decades, behavioral data from various taxa provide evidence consistent with the genetic coupling hypothesis by studying male signals and female preferences in interspecific hybrids (Hoy et al., 1977; Doherty and Gerhardt, 1984; Ritchie, 1992; Shaw et al., 2007; Shaw and Lesnick, 2009). Several other studies have reported contrary empirical results (Boake, 1991; von Helversen and von Helversen, 1975; Löfstedt et al., 1989) or questioned the interpretation of the hybrid studies that supported the genetic coupling hypothesis (Butlin and Ritchie, 1989). For example, assuming that both

traits vary quantitatively and are under polygenic control, interspecific hybrids would be expected to have calls and preferences with values intermediate between those of the parental species. Hence senders and receivers would be matched to some extent, even if the genes controlling call production and recognition were entirely independent (Butlin and Ritchie, 1989). This scenario could be complicated if one or more of the genes controlling these processes were sex linked, but Hoy and Paul (1973) found that females of each reciprocal cross between two species of crickets preferred the distinctive songs of males of the same cross.

The essence of the genetic coupling hypothesis is that relatively few genes dominate in the control of both signals and preferences. One hypothesis is that they might determine the properties of a common neural oscillator that dictates the timing of sound production in the signaler and simultaneously serves as a reference oscillator for the incoming stream of neural spikes occurring in the female auditory system (Alexander, 1962). Evidence for such a "resonance" mechanism on the receiver side has been found in behavioral studies of katydids and other animals (Bush and Schul, 2006; Hennig, 2003). However, the demonstration that sound producing and recognizing neural mechanisms are separately located in orthopteran insects (e.g., Bauer and Helversen, 1987) indicates that a single physical mechanism postulated by Alexander (1962) cannot apply to this system.

Nevertheless, common genes, or tightly linked genes, could still facilitate the coordination of the development and expression of sender and receiver mechanisms that would change in a parallel fashion during periods of evolutionary divergence. Evidence for this hypothesis is available from studies of Hawaiian crickets (Shaw and Lesnick 2009) and

Heliconus butterflies (Kronforst et al., 2006). These studies also offer evidence that coordination between senders and receivers are maintained primarily by linkage disequilibrium of multiple genes (e.g., Bakker and Pomiankowski, 1995) and by common or tightly linked genes located on the same part of a chromosome.This evidence will be reviewed in the Discussion.

North American hylid frogs have several advantages as subjects for research concerned with the mechanisms and evolution of acoustic communication. First, the acoustic signals of males are unlearned and stereotyped, allowing objective and unequivocal characterization and quantification of species-specific properties (Gerhardt and Huber 2002). The analysis of acoustic signals recorded within about 2 m of calling male frog almost certainly provides information about the physical properties that females can assess before making a mate choice. By contrast, the perception of visual signals depends on lighting conditions and angle of view, and the transmission of chemical signals is subject to uncertain wind or currents. Second, females that are ready to mate nearly always initiate sexual contact with a calling male. Such females reliably show phonotaxis toward speakers that emit pre-recorded conspecific calls, thus demonstrating that visual, olfactory, and other sensory cues are superfluous. Third, acoustic stimuli that are as effective as pre-recorded conspecific call can be easily synthesized. Playback of such signals in which particular properties are systematically varied allows researchers to identify the key features that females use for call recognition and the most effective values of those properties. (Gerhardt, 1978; Gerhardt, 1982; Bush et al., 2002). Finally, several pairs of North American hylids show high levels of genetic compatibility, and viable hybrids have

been found in the wild. Studies of the calls and preferences of lab-reared interspecific hybrids can set the stage for analyses that can provide insights about the genetic architecture underlying these traits. The main disadvantage of this system is that male hybrid offspring require about one year and females about two years to reach sexual maturity (Doherty and Gerhardt 1984; this study).

Doherty and Gerhardt (1984) raised interspecific hybrids between two treefrogs to sexual maturity and studied both the calls of F_1 males and preferences of F_1 females. Female hybrids preferred hybrid calls to those of the parental species, provided that the acoustic energy of alternative sounds was equivalent. Indeed, the use of synthetic calls was required to achieve this equivalence because the temporal organization of pulses in the calls of the two parental species (*Hyla chrysoscelis* and *H. femoralis*) and their hybrids was so different. Subsequently, phylogenetic analyses showed that the species were much more distantly related than previously thought. For all of these reasons, and the fact that only a few sexually mature hybrids were available, no F_2 or backcrosses were attempted.

The goal of this research was to determine the pattern of inheritance of species-specific signal characters (temporal and spectral) and corresponding female preferences for those signals in the North American treefrogs *Hyla chrysoscelis* and *H. avivoca,* sister species belonging to the *versicolor* group of Holoarctic *Hyla*. *H. chrysoscelis* has a vast geographic range extending from the southeastern and central US northward through parts of south-central Canada, while *H. avivoca* has a more limited range in the southeastern US. *H. chrysoscelis* where it is broadly sympatric with *H. chrysoscelis*. Because they often breed at the same time in swamps in riparian habitats, there is the potential for interspecific

mating, and natural hybrids have been tentatively identified on the basis of their intermediate calls and morphology (Gerhardt, 1974). We investigated signal and preference inheritance in these species because they are not only more closely related than *H. femoralis* and *H. chrysoscelis*, but also because the temporal organization of sound pulses is qualitatively similar. The spectral structure of male calls and the acoustic criteria underlying female preference differ between these two species qualitatively, allowing us to potentially demonstrate dominance effects in the F_1 and perhaps simplify the interpretation of the results of the F_2 and backcross procedures that might distinguish between phenotypes based on multiple genes of small effect, a few genes of large effect, and to estimate the extent of matching between male signal properties and female preferences. (Gerhardt, 1974; Hoy et al., 1977; Doherty and Gerhardt, 1984; Kronforst et al., 2006; Shaw and Lesnick, 2009).

Males of both species produce calls composed of trains of pulses (trills) at a relatively constant rate; the pulse period within these trills is even more stereotyped, especially in *H. chrysoscelis*. Females of *H. chrysoscelis* show strong preference for conspecific pulse rate, which ranges from 34-49 pulses/sec based on geographic location (Gerhardt, 1994). *H. avivoca* males produce pulses at a much slower rate (about 8 pulses/sec). Pulse shape also differs between the two species, with *H. chrysoscelis* producing pulses with fast, logarithmic rise times and *H. avivoca* males producing pulses with a slower, linear rise time. *H. chrysoscelis* do not discriminate between conspecific and heterospecific (*H. versicolor*) pulse shape when the pulses are of typical duration (10 ms), but show a strong preference for conspecific pulse shape when the signals were longer in

duration (Gerhardt, 2005). Martinez-Rivera and Gerhardt (2008) provide evidence that females of *H. avivoca* discriminate potential mates based on minimum pulse duration because females show a strong preference for non-overlapping pulses, suggesting pulse rate is not the main female-preference criterion. Unlike females of *H. chrysoscelis*, which use pulse rate alone for species recognition (Schul and Bush, 2002), in tests of overlapping signals, females of *H. avivoca* prefer calls with longer-than-average intervals (lower pulse rate) resulting from active modification of the intervals by nearby males, which interdigitate pulses in order to avoid masking (Martínez-Rivera and Gerhardt 2008).

In addition to fine-scale temporal differences, the calls of the two species also differ spectrally. Both species have dominant frequencies (DF) between 2.0 and 2.6 kHz, but calls of *H. chrysoscelis* also have a secondary (fundamental frequency) peak at about one-half the frequency of the DF. Females of *H. chrysoscelis* prefer calls with both bands to the alternative with a single band of either low or high-frequency (Gerhardt et al 2007). By contrast, a second, lower-frequency band is absent in *H. avivoca,* and the DF corresponds to the fundamental frequency. Weak (> -30 dB) higher component (harmonics) are present, but they are almost certainly above the effective hearing range of this species. Furthermore, females of *H. avivoca* discriminate against synthetic calls that contain a secondary band that is an octave lower in frequency in favor of calls with a single band at the dominant frequency (Gerhardt et al., 2007; Martínez-Rivera and Gerhardt 2008). By contrast, females of *H. chrysoscelis* prefer calls with both bands (Gerhardt et al., 2007). Such a secondary band would be highly audible to females of *H. avivoca.*

METHODS

Analysis of the Advertisement Calls of H. chrysoscelis and H. avivoca

We recorded the advertisement calls of five *H. avivoca* males from Heron Pond in Johnson County, IL (USA) during the summer of 2012. We were unable to locate calling *H. chrysoscelis* in this location during our recording trip; therefore we used call data from five individuals in a nearby population in Phelps County Missouri. For each individual, we analyzed five separate calls using a solid state Marantz digital recorder (PMD670) and a Sennheiser directional microphone (ME-66) positioned 45-60 cm from the male. We recorded the air and water temperature data to the nearest 0.1°. Calls were digitized at a sampling grate of 48 kHz and analyzed using Adobe Audition v2.0 (Adobe Systems Inc., San Jose, CA, U.S.A). We analyzed variability of call traits using R statistical software (Team, 2013).

Generation of Hybrid Population

We produced an F_1 population via an artificial cross between two male *Hyla chrysoscelis* and a female *H. avivoca*. We collected an amplexed pair of *H. avivoca* and two calling *H. chrysoscelis* males from Johnson County, IL (USA). The *H. avivoca* pair was separated and the eggs were manually extracted from the female and divided into two petri dishes containing Holtfreter's solution. We sacrificed both *H. chrysoscelis* males via pithing and removed a testis from each individual. A single testis was crushed in solution with the eggs and mixed to allow maximum fertilization. Several attempts to make reciprocal crosses failed. In most cases fertilization failed or fertilized clutches perished after

hatching. We do not know if these failures were attributable to genetic incompatibilities in this reciprocal cross or whether there were problems with eggs or sperm of the individuals we used.

Fertilized clutches of the two parental cohorts were separated into several small containers until hatching. Clutches were monitored daily and dead embryos were removed. Tadpoles from the two cohorts were transferred to separate outdoor 800 l tanks to continue development. Tanks were inoculated with leaf litter from the collection location of the parental species. We retrieved individuals after metamorphosis (between 25-45 days) and housed them in individual containers in the laboratory. Damp peat moss covered with a cloth provided a damp environment for the animals. Individuals were fed fruit flies for the first several months and small crickets thereafter. Males reached sexual maturity about 9 months after hatching, and females reached sexual maturity after 22 months. After sexual maturity individuals from the same parental cross were maintained in group housing. All individuals used in the analyses were toe clipped before behavioral trials.

Recording F_1 Advertisement Calls

Individual male calls were recorded on a nightly basis using Adobe Audition software (v 2.0 Adobe Systems Inc., San Jose, CA, USA). We placed a single focal male in a small (1 m x 0.5 m) enclosed arena crafted with anechoic foam blocks. Inside the arena, the caged male was placed on a platform 10 cm in front of a Macintosh laptop. Recordings were made using the built in microphone. To stimulate calling behavior, we played a series of natural hybrid calls at the onset of each trial. The software was programmed to

automatically record sounds above a certain minimum threshold which enabled us to record only when the focal male was calling. Because pulse rates are affected by body temperature, deep body temperature measurements were taken and all call data were corrected to 20°C using the results of a temperature regression analysis of pulse rate in our sample of calling hybrids.

Calls were analyzed using Raven sound analysis software v.1.2.1 (Cornell Lab of Ornithology, 2003). Pulse rate was the reciprocal of the pulse period (time from beginning of one pulse to the beginning of the following pulse), and we measured each pulse period from five calls of every individual. Pulse rise time was measured from amplitude versus time displays and defined as the time from the point at which the peak-to-peak amplitude was 10% above the baseline to the time when it reached its maximum peak-to-peak amplitude. Spectral properties were measured from power spectra (Hamming window, fast Fourier transform length = 1024 samples). We measured the frequencies with the highest relative amplitude in both the high- and (if present) the low-frequency range.

Stimulus Design for Female Preference Tests

We designed five artificial stimuli using custom software (Schwartz, unpublished) based on the two parental species and the hybrid call (Fig. 4). We used the mean values of the temporal and spectral features of representative *H. avivoca* and *H. chrysoscelis* calls recorded in the field (Table 3). For the hybrid stimuli, we created three stimuli using the average temporal and spectral values of 15 calling males. The first stimulus consisted of a 28-pulse call containing both the dominant frequency peak at 1.6 kHz and a secondary

peak at 3.3 kHz (-9 dB relative to the dominant frequency). The second and third hybrid stimuli were temporally identical to the standard, but either the dominant or secondary frequency was digitally removed to create stimuli containing only the low or high frequency peak.

Phonotaxis Experiments

Mature F_1 females were hormonally primed with progesterone and prostaglandin to stimulate sexual receptivity (Gordon and Gerhardt, 2009). After priming, females acclimated in a temperature-regulated incubator until testing. Females were tested in a dark anechoic chamber maintained at 20°C. We used a Larsen Davis 800D sound level meter to adjust the sound level to 85 dB (re 20 μPa, fast RMS (root-mean-square) C-weighted) at the release location midway (1 meter) between two speakers, where the females were initially released.

After three repetitions of each stimulus, the female was released remotely and her movements observed remotely via an infrared-sensitive camera and television monitor. We recorded a response if the female exhibited phonotaxis and moved within 10 cm of either speaker. Gordon and Gerhardt (2009) reported that artificially primed females exhibit the same phonotactic choice as their natural counterparts, however, responses are usually slower. Therefore, females were given a total of 10 minutes to make a choice, after which a "no choice" was scored. Each female was tested only once per stimulus pair, and all females received at least a five minute time out between trials. All procedures were

approved by the University of Missouri Animal Care and Use Committee (protocol number 1910).

We performed a two-tailed binomial test for each set of choice tests. The figures below illustrate the proportion of females choosing the standard, two-peaked hybrid call over the alternative stimulus (single-peaked hybrid, *H chrysoscelis*, or *H. avivoca*). The error bars are 95% credible limits on these proportions. Because we assumed a uniform prior distribution, these intervals correspond numerically to confidence limits but have a more straightforward interpretation (see Gerhardt 2005). These tests lack strict independence because several females responded in multiple tests; thus, we did not conduct any global statistical procedures. For each test (one response per female), we assume that there was no carryover effect from a previous different test, an assumption supported by data in Gerhardt and Doherty (1988).

RESULTS

Survivorship and Sample Size of Hybrids

A total of 491 tadpoles were transferred to indoor housing in August of 2011. Survivorship was about 50% after one year. Males were heard calling 9 months after being transferred and females tested positively for sexual receptivity after 22 months. A majority of the surviving male population never produced acoustic signals. About 10% of the total adult population showed signs of developmental abnormalities and suffered higher mortality rates relative to the rest of the population. We analyzed the calls of 15 males that

readily called during our recording sessions and the 15 females that responded in phonotaxis trials.

Call Characterization: Temporal Traits

Hybrids produced pulses that were organized into short trains that were repeated at a regular rate (Figure 2-1). Variability within and between the two parental cohorts was minimal (two-tailed test: $p > 0.05$ for all call traits, Table 2-1); therefore we present the pooled results of the call analysis below. The mean values of the gross and fine-temporal properties across all 15 individuals are summarized in Table 2-1. Across all individuals, pulse duration, pulse number, and call rate fell between the two parental species, but closer to *H. chrysoscelis* (Table 2-2). Call rate was variable both between and within individuals. Most individuals (10 of 15) produced calls between 1.8 and 2.4 seconds in length (30-40 pulses/call). One male produced much shorter calls relative to the other males (Male 5: Fig. 2-2d, Table 2-1). Most calls gradually increased in amplitude over the first few pulses before reaching maximum amplitude for the remainder of the call (see Fig. 2-2e). Otherwise, call rise times were linear and proportional to call duration (i.e. shorter calls has faster rise times than longer calls). Pulse rate was much slower than in *H. chrysoscelis*, falling closer to rates produced by *H. avivoca*.

Call Characterization: Spectral Features

Compared to both parental species, the signals of the hybrids were more like those of *H. chrysoscelis* in that there were two prominent spectral peaks (Fig. 2-3, Table 2-3). The lower-frequency peak averaged about 1.6 kHz, or about 0.4 kHz higher than the typical

frequency of the analogous band in *H. chrysoscelis* calls. The high-frequency peak, which was harmonically related, averaged about 3.3 kHz, compared the 2.6 kHz in typical calls of *H. chrysoscelis*. Whereas the relative amplitude of the high-frequency band is usually 6-12 dB higher than that of the low-frequency band in *H. chrysoscelis*, the low-frequency band in hybrid calls had a higher (about 8 dB) relative amplitude. Calls of *H. avivoca* have nearly all of the sound energy concentrated in a single peak at about 2.6 kHz; the relative amplitude of second harmonics in these calls are typically of the order of -30 to -40 dB and could even be distortion products caused by overloading microphones, recorders or input amplifiers of the sound analysis equipment.

Hybrid Female Preferences

The artificial stimuli used in the female preference tests are shown in Fig. 2-4. The results of the four tests are shown in Fig. 2-5. Females did not show strong discrimination against the hybrid stimuli containing only the high or low frequency peak (two-tailed binomial test: $p > 0.05$ for both tests). Females significantly preferred the calls of the maternal species, *H. avivoca,* over the standard, two-peaked hybrid signal (two-tailed binomial test: $p = 0.034$). A majority of females chose the standard two-peaked hybrid signal over the *H. chrysoscelis* stimulus (two-tailed binomial test: $p = 0.057$). The response times of female hybrids was slower than typical responses of wild type individuals; however, our results were similar to those of hormonally primed hybrids of *H. femoralis* and *H. chrysoscelis.* The mean response time 481 seconds (N=48, range = 103-1251

seconds), compared to a mean of 467 seconds in Doherty's (1982) study (N= 60, range = 89-1265 seconds).

DISCUSSION

The goal of this study was to characterize the behavioral phenotypes of F_1 males and females in order to frame further studies concerned with the genetic architecture underlying sexual signals and preferences in behaviorally isolated sister species. While our results do not allow for a conclusive report on the number of genes involved or the genetic interactions at play, our data provide some important insights. First, F_1 male call traits varied, with some falling closer to the average values of one parental species (though still outside the normal range for each species), and others showing intermediate values. Our results suggest that these signal features are likely due to the additive effects of polygenic inheritance with the potential for sex-linkage for certain traits. Interestingly, all calling individuals produced signals with two frequency peaks, which is qualitatively more similar to the paternal species, *H. chrysoscelis*. Reciprocal crosses are necessary to determine whether the expressed spectral phenotype is due to sex linkage, dominance, or some other genetic mechanism.

Female preference was not restricted to the phenotype of their male siblings, as would be expected under the genetic coupling hypothesis. While females did respond positively to hybrid stimuli, they also showed a significant preference towards the maternal (*H. avivoca*) stimulus and significant discrimination against the paternal (*H. chrysoscelis*) stimulus. Thus female preference is probably regulated by multiple genetic

factors rather than a single gene. Additional research is required to assess the possibility of dominance components in a robust fashion. While we did not test preference strength in this study, female preference is clearly more variable in the hybrids than in either of the parental species. Moreover, females were relatively indifferent to the spectral makeup of hybrid male calls, and even preferred the signals of their maternal species to those of hybrids. There is the potential for distinct phenotypic classes in this trait in which some individuals show preferences for one trait (e.g. a high frequency component) and others do not. This could arise due to differences in regulatory genes or gene dosage effects influencing the receptivity to certain temporal or spectral traits. In this case we might expect, for example, females with strong discrimination against a low frequency component (as is seen in females of *H. avivoca)* to prefer the two-peaked hybrid call over the single high-peak alternatives as well as the paternal call which contains a high frequency component. Due to low sample sizes and the fact that we were unable to gather responses to every test in each female, we cannot draw such conclusions at this time. Nevertheless, the differences in male and female phenotype are clear evidence that these behaviors are not tightly linked.

Several other studies have investigated the genetic inheritance of sexual signals and preferences via behavioral and genetic analyses. A few lend some support to the genetic coupling hypothesis. Studies of *Teleogryllus* crickets by Hoy and his colleagues provided some of the first behavioral evidence to support the genetic coupling hypothesis (Hoy and Paul, 1973; Hoy et al., 1977). In reciprocal crosses between closely related crickets (*Teleogryllus oceanicus and T. commodus)*, they found that hybrid males produced distinct

calls differing both temporally and spectrally than the parental calls, and hybrid females preferred the calls of their brothers over the parental calls. These authors speculated that the coordination was due to linked neural oscillators. Doherty and Gerhardt (1984) crossed two different species of hylids and found strong behavioral support for linkage between the temporal features of the male signal and the preferences of hybrid females. Two recent studies in insects provide behavioral support for the coupling hypothesis in both a visual and acoustic communication system.

Shaw and Lesnick (2009) and Kronforst et al. (2006) were able to take advantage of modern genetic techniques to identify and map the genetic loci controlling the behaviors in question, allowing them to speculate on the underlying genetic basis controlling the behavioral phenotypes. Shaw and Lesnick (2009) used QTL analysis to show strong support for tight linkage for song type and female preference between closely related species of Hawaiian crickets (genus *Laupala)*. Kronforst et al. (2006) crossed closely related sympatric species of *Heliconius* butterflies and found that the visual cue and male mate preference in the hybrids closely matched. As in the Hawaiian cricket study, QTL data supported their behavioral results. These studies have two common features which may elucidate circumstances in which genetic coupling may play a role in the coordination of signals and preferences. First, both studies investigated species considered to have evolved rapidly. Second, mate choice decisions are based on a single, relatively simple phenotypic trait (wing color in *Heliconius* and pulse repetition rate in *Laupala).*

In contrast, results from several behavioral and genetic studies suggest that some sexual traits and preferences for those traits do not correspond between male and female

conspecifics (Boake, 1991; Löfstedt et al., 1989; Ritchie, 2000; Rosenthal et al., 2003). Rosenthal et al (2003) investigated the inheritance of male morphology and behavior and female preference in swordtails. Surprisingly, their data indicate a high degree of variability in the distribution of hybrid male visual phenotypes and weak hybrid female preferences for specific phenotypes in natural hybrids. Our results show similar patterns with regard to female preference and variation in male signal display, suggesting these behavioral traits are unlikely to be under common genetic regulation.

Future Directions

Several generations of reciprocal crosses are necessary to clarify the role, if any, of sex-linked genes in the inheritance of the signal and preference traits in *H. chrysoscelis* and *H. avivoca*. Researchers have successfully produced multiple generations of insects, but few have quantified the distribution of signal and preference in phenotypes in F_2 and backcrossed vertebrates. Additionally, uncovering the specific mechanism(s) underlying female preference in hybrids is essential because such qualitative shifts in female preference may provide critical information regarding behavioral divergence between different species or incipient species (Schul and Bush 2002). Females of *H. avivoca* and *H. chrysoscelis* have distinct recognition mechanisms and additional experiments utilizing unequal playback amplitudes are needed to systematically test the strength of preference for each trait in interspecific hybrids. Future experiments should test female preference across the range of variation in both the hybrid and parental populations to determine if preference functions shift from one generation to the next. Finally, the next step in this

research is to begin searching for candidate loci upon which to investigate using QTL analysis to support this important behavioral work.

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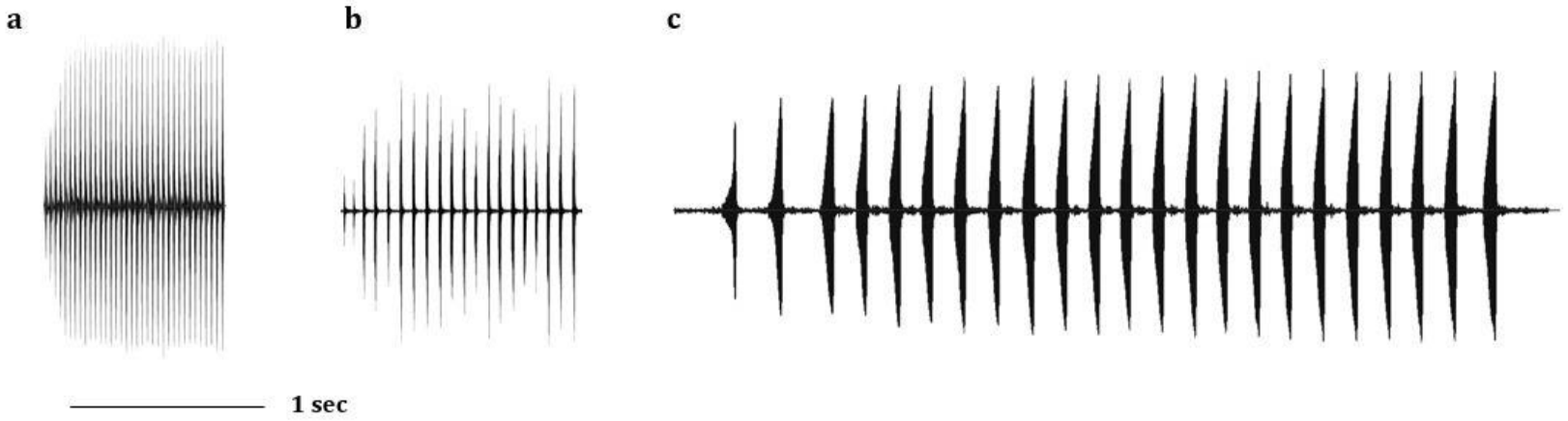

Figure 2-1. Oscillograms of hybrid and parental calls

Oscillograms of (a) typical *H. chrysoscelis* (paternal species), (b) a representative F_1 hybrid, and (c) typical *H. avivoca* (maternal) advertisement call. As in both parental species, hybrids produced calls consisting of pulses organized into discrete trains, though temporal traits differed markedly from the parental values.

Male (cohort)	Pulse Duration (ms)	Pulse Rate (pulses/sec)	Pulse Number	Call Duration (ms)	Call Rate (calls/min)
1 (P1)	25 (4)	11.8 (0.6)	23.0 (0.7)	1690 (65.7)	17 (1.1)
2 (P2)	25 (3)	12.5 (1.2)	34.4 (0.5)	2490 (70.6)	13 (0.8)
3 (P1)	21 (2)	13.3 (1.0)	35.4 (0.5)	2380 (12.8)	15 (1.0)
4 (P2)	20 (2)	16.7 (0.7)	40.6 (0.5)	2450 (76.1)	11 (0.7)
5 (P2)	26 (7)	15.5 (1.3)	18.6 (0.5)	1400 (37.6)	13 (0.9)
6 (P1)	20 (2)	15.2 (1.2)	36.4 (0.5)	2260 (75.0)	13 (0.9)
7 (P2)	19 (2)	15.5 (1.9)	28 (0.7)	1670 (16.6)	14 (0.9)
8 (P2)	19 (2)	15.8 (1.2)	33.4 (0.5)	2003 (17.4)	14 (0.9)
9 (P2)	19 (1)	19.3 (0.9)	34.4 (0.5)	2080 (40.1)	19 (1.2)
10 (P2)	20 (1)	14.1 (0.3)	35.2 (0.4)	2174 (15.0)	12 (0.8)
11 (P1)	20 (1)	15.2 (0.8)	30.4 (0.5)	1864 (17.5)	13 (0.9)
12 (P1)	23 (1)	15.8 (0.8)	31.2 (0.8)	2184 (59.6)	17 (1.3)
13 (P1)	23 (1)	15.8 (0.7)	27.8 (0.4)	1963 (42.5)	13 (0.8)
14 (P2)	22 (1)	15.6 (0.4)	32.8 (0.4)	2322 (12.7)	12 (0.8)
15 (P2)	22 (2)	14.6 (0.8)	35.4 (0.5)	2314 (15.5)	18 (1.2)
Grand mean	22 (2)	15.2 (178)	31.8 (5.6)	2082 (317)	14.13 (2.4)

Table 2-1. Temporal traits of hybrid calls

Breakdown of temporal traits across two cohorts (P1 and P2) of F_1 hybrids. The values shown are means with standard deviations in parentheses. Two-tailed t-tests revealed no significant difference between the two cohorts (pulse duration: p=0.522; pulse rate: p=0.302; pulse number: p=0.554; call duration: p=0.806; call rate: p=0.592).

	Pulse Duration (ms)	Pulse Rate (pulses/sec)	Pulse Number	Call Duration (ms)	Call Rate (calls/min)	Dominant Frequency (kHz)	Secondary Frequency (kHz)
H. CH	9 (1)	56.06 (1.5)	35.68 (4.7)	.638 (0.094)	19.43 (1.98)	1.2 (0.05)	2.6 (0.05)
Hybrid (pooled)	22 (2)	15.12 (1.78)	31.80 (5.6)	2080 (317.26)	14.14 (2.38)	1.6 (0.06)	3.3 (0.05)
H. AV	66 (5.5)	5.95 (0.85)	21.73 (3.44)	3640 (0.718)	5.27 (2.32)	2.6 (0.04)	N/A

Table 2-2. Summary of hybrid and parental call traits

Summary of temperature-corrected call trait data for the hybrid (N = 15) and parental (N = 5 males per species). Values are means and standard deviations shown in parentheses. The secondary peak in the hybrid call is -9 dB below the dominant frequency (SD = 1.3 dB). For *H. chrysoscelis* the secondary band is -6 dB below the dominant frequency (SD = 0.8 dB). Note: *H. avivoca* does produce energy at a secondary frequency, however the relative amplitude is below the threshold for biological relevance.

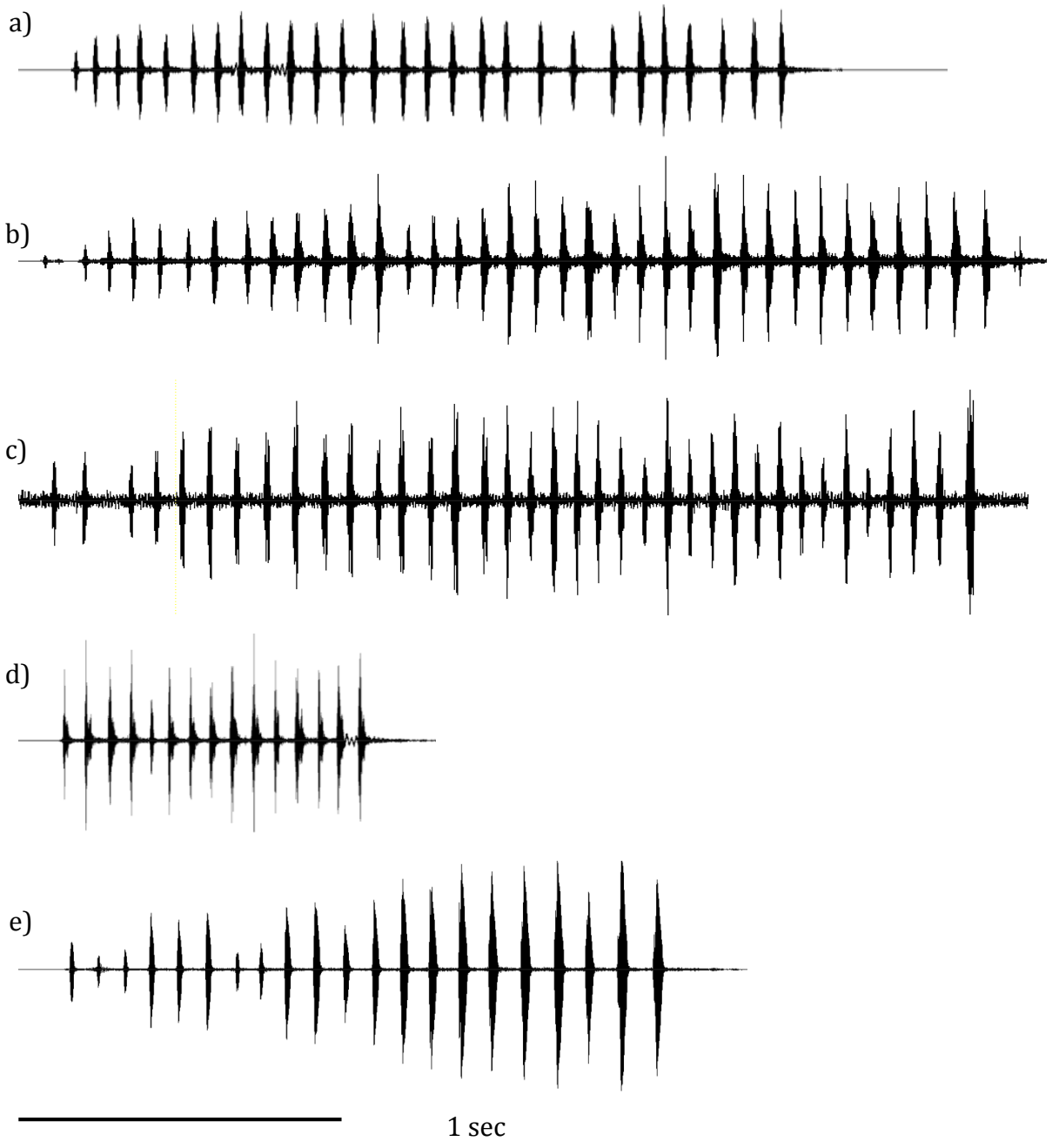
a)
b)
c)
d)
e)
1 sec

Figure 2-2. Example hybrid calls

Representative oscillograms from calling hybrid males. (a-c) represent typical calls (n=12), (d) atypical short call recorded in one individual, (e) example of gradual amplitude modulation throughout the duration of the call.

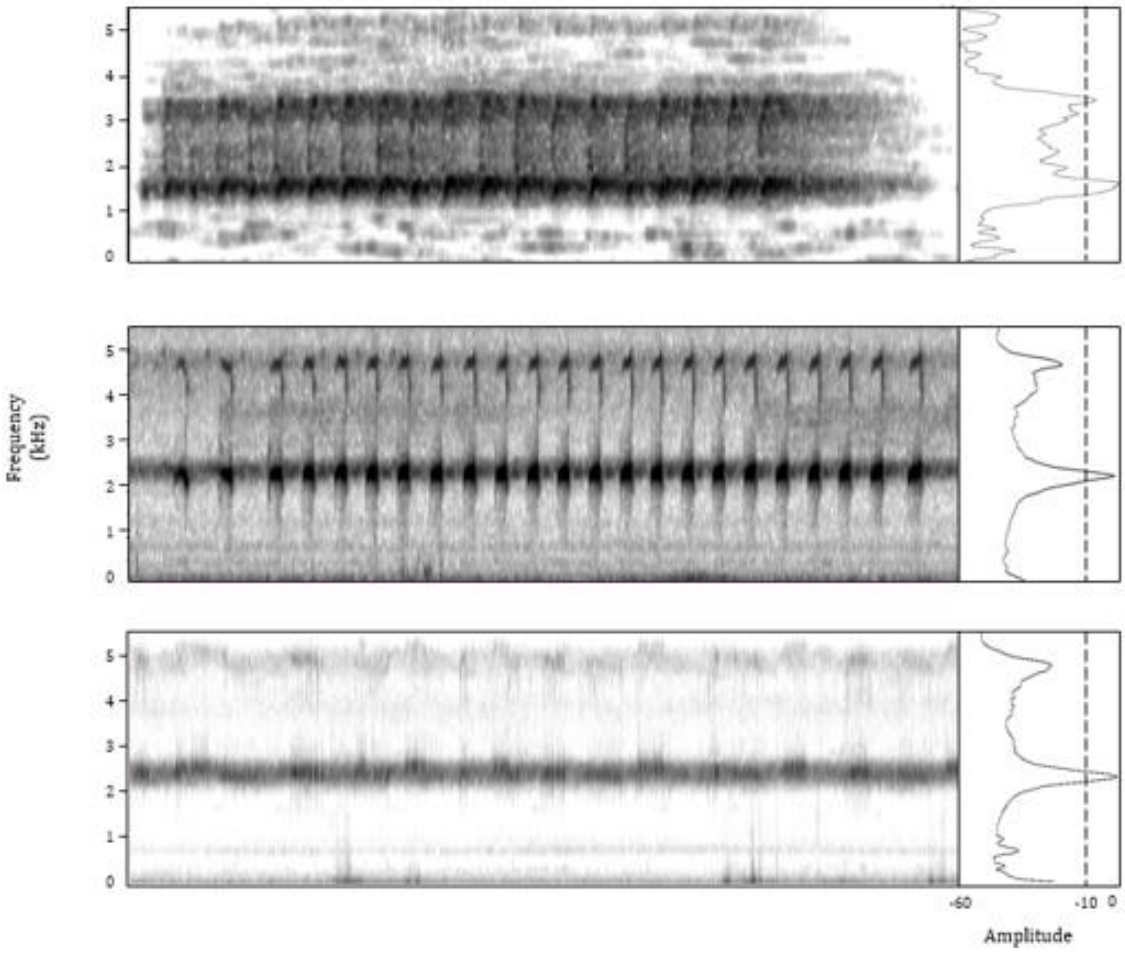

Figure 2-3. Spectral properties of hybrid and parental calls

Spectrogram and power analysis of representative hybrid (top trace) and parental calls (middle trace = *H. avivoca,* bottom trace = *H. chrysoscelis)*. Hybrid individuals produced signals with two frequency peaks, similar to that of *H. chrysoscelis.* The second band seen in *H. avivoca* is approximately -20 dB lower than the dominant frequency and is unlikely to be biologically relevant.

Male (cohort)	Dominant Frequency (kHz)	Secondary Frequency (kHz)	Relative Amplitude (dB)
1 (P1)	1.6 (0.07)	3.3 (0.05)	-9 (-10, -8)
2 (P2)	1.7 (0.06)	3.2 (0.05)	-15 (-15, -14)
3 (P1)	1.6 (0.09)	3.5 (0.13)	-14 (-14, -13)
4 (P2)	1.7 (0.08)	3.3 (0.05)	-9 (-9,-8)
5 (P2)	1.6 (.05)	3.3 (0.06)	-9 (-9,-8)
6 (P1)	1.6 (0.05)	3.2 (0.05)	-9 (-9,-8)
7 (P2)	1.7 (0.05)	3.3 (0.05)	-6 (-6, -5)
8 (P2)	1.6 (0.05)	3.3 (0.05)	-5 (-6, -5)
9 (P2)	1.6 (0.09)	3.3 (0.08)	-9 (-9,-8)
10 (P2)	1.7 (0.09)	3.4 (0.05)	-10 (-10, -9))
11 (P1)	1.6 (0)	3.3 (0.05)	-9 (-9, -8)
12 (P1)	1.7 (0.05)	3.3 (0.09)	-6 (-7, -6)
13 (P1)	1.6 (0.07)	3.3 (0)	-6 (-7, -6)
14 (P2)	1.6 (0.05)	3.3 (0.05)	-8 (-9, -8)
15 (P2)	1.6 (0.05)	3.4 (0)	-10 (-10, -10)
Grand Mean	1.62(0.06)	3.3 (0.05)	-8.6 (-14.8, -5.2)

Table 2-3. Spectral properties of hybrid calls

Breakdown of spectral traits across two cohorts of F_1 hybrids. The frequency values are means with standard deviations in shown in parentheses. Relative amplitude values are medians and range in parentheses. There was no significant difference between the calls of males belonging to either group.

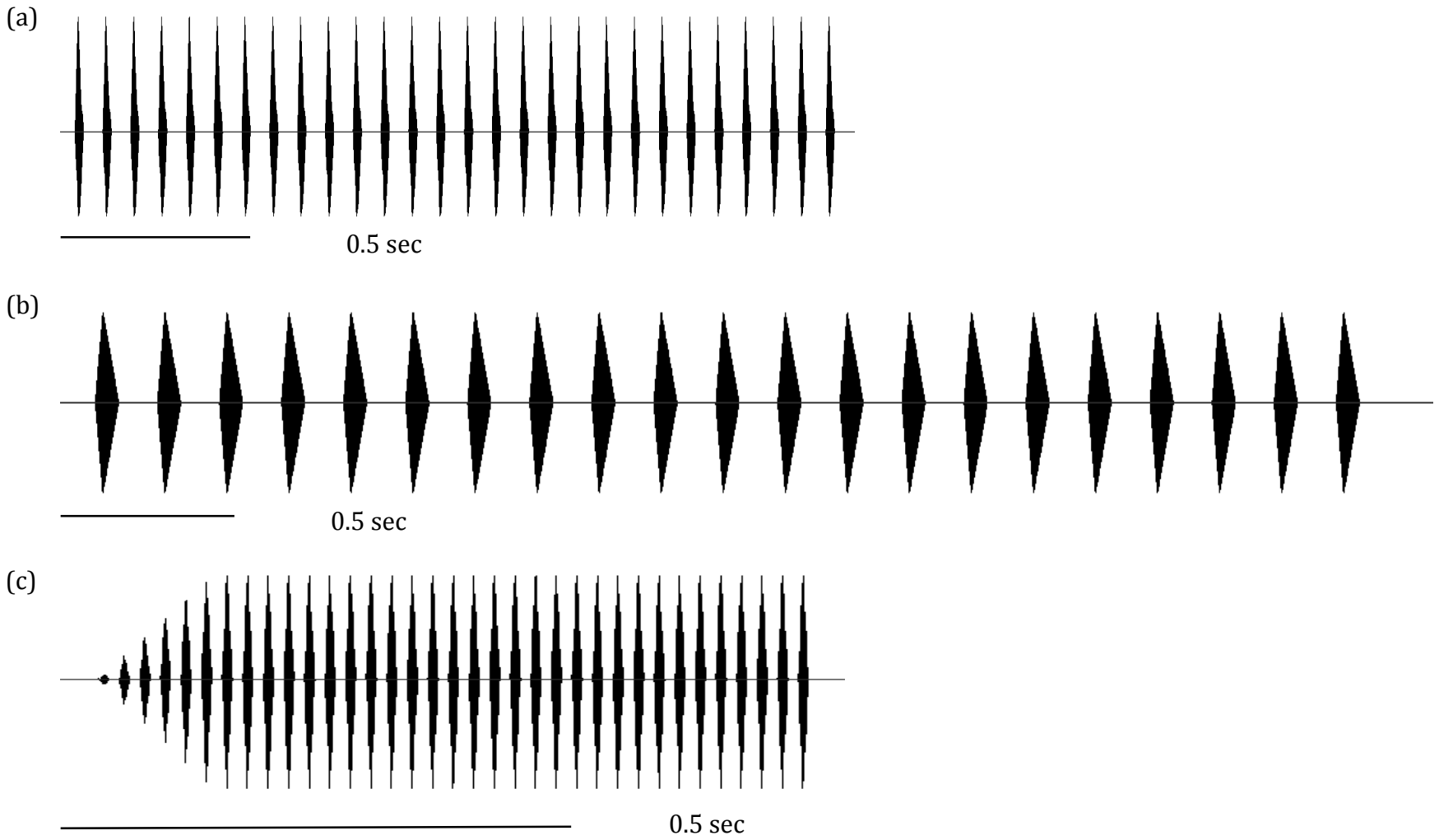
(a)
0.5 sec
(b)
0.5 sec
(c)
0.5 sec

Figure 2-4. Artificial stimuli for female preferences tests

(a) Artificial hybrid call, (b) artificial *H. avivoca* call, (c) artificial *H. chrysoscelis* call. Note differences in time scale.

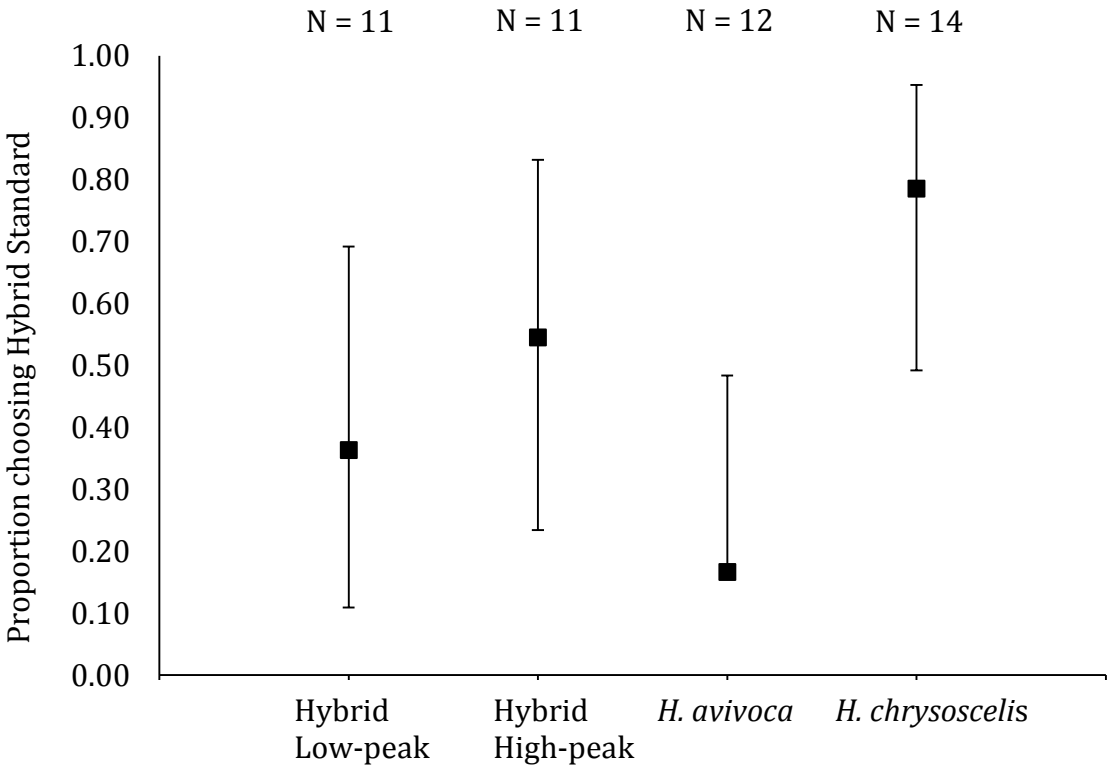

Figure 2-5. Preferences of hybrid females

Error bas are 95% credible intervals. Females did not discriminate between hybrid stimuli containing only the high or low frequency peak, but showed significant preference for the calls of *H. avivoca,* and against *H. chrysoscelis.*

CHAPTER 3

DYNAMIC SIGNALLING STRATEGIES IN THE PINE WOODS TREEFROG (HYLA FEMORALIS): SOCIAL CORRELATES OF SIGNAL PLASTICITY

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INTRODUCTION

For many species, pair formation depends on the production of advertisement signals by one sex and the appropriate behavioral response by the other (Andersson, 1994). In lekking species, intense competition can drive individuals to implement signaling strategies to increase their ability to obtain mates. These often involve adjusting the frequency, duration, or intensity of those signals, or switching to distinctive aggressive signals, in order to out-compete or repel neighboring rivals (Bradbury, 1983; Wells and Schwartz, 1984a). Signalers often respond to changes in the level of competition with graded aggressive responses and adjustments in signal timing relative to close neighbors (Bee, 2003). The prevalence of such adjustments in signal timing is documented across a wide variety of taxa and signal modalities (Fischer et al., 2002; Lim and Greenfield, 2007; Zelick and Narins, 1985; Brumm, 2006). Signal timing between neighboring callers falls along a continuum between perfect synchrony (signals occur in phase with another) and perfect alternation (signals occur 180 degrees out of phase) (Greenfield, 1994).

Although context-dependent plasticity may drive individuals to adjust their signals, plasticity is limited by physiological, neural, and environmental constraints such as body size and body temperature (Ryan, 1998), as well as selective pressures which have shaped the signal phenotypes within a species (Searcy and Andersson, 1986; Slabbekoorn and Smith, 2002). Sexual selection via mate choice is commonly documented as a strong driver of signal variability. For example, Gerhardt (1991) hypothesized that what he termed dynamic properties, with high within-individual variation, may provide specific information to females about the physical or genetic quality of potential mates. By contrast,

static properties, with low within-individual variation, may confer information about species or individual identity. The first possibility is likely if among-individual variability is also limited; the second, if there is substantial among-individual variability (e.g. Bee et al., 2001). It follows that dynamic properties are likely to be under directional selection by females, whereas those static traits that convey reliable information about species or perhaps population identity are likely to be under stabilizing or weakly directional selection (also see Gerhardt, 1982; Bush et al., 2002). The signals of individuals thus consist of a composite of properties that results from some balance between these various forces.

We investigated acoustic communication in a North American hylid, the pine woods treefrog (*Hyla femoralis)*. Its advertisement call has been historically characterized by long trains of pulses repeating at a highly irregular rate of 6 to 12 pulses per second (Fig. 1). Among North American frogs, and especially the hylids, such pulses usually show little within-individual variability. *H. femoralis* is unique in that the advertisement signals of individuals are highly plastic. Furthermore, these pulses are not organized into discrete trains, or calls in the usual terminology. The aggressive signals of the Hourglass treefrog *(H. ebraccata),* share some of these attributes (Wells and Schwartz, 1984a), and cricket frogs (*Acris crepitans)* produce pulse groups at somewhat irregular intervals (Wagner, 1989). Another species that is somewhat closely related to *H. femoralis,* the bird-voiced treefrog *H. avivoca*, shows somewhat higher-than-typical within-male variation in pulse period (the reciprocal of pulse rate) within the call (trill) when competitors are nearby. Martinez-Rivera and Gerhardt (2008) argue for the potential of cooperative signaling in *H. avivoca* because a pair of nearby males frequently inter-digitate their pulses within trills. This

behavioral plasticity could be a mechanism by which males minimize acoustic overlap. Behavior consistent with this explanation has also been observed in the more distantly related hylid, *H. microcephala* (Schwartz and Wells, 1985). The within-male variability of *H. femoralis* is, however, appreciably greater than in *H. avivoca* and *H. microcephala*, regardless of the social context. Understanding the patterns underlying the unique calling patterns in this species and the consequences for mate attraction (see Chapter 4) can offer new insights about the underlying mechanisms and evolution of vocal competition.

Our approach to this system centered on documenting the variability of pulse rate in *H. femoralis*, both during solo calling and in response to hearing the calls of other males or to playbacks of synthetic signals. We could then quantify how males reacted to external acoustic signals and describe the resulting timing relationships between the pulses of focal males and those of the external signals. If male-male competition plays a role in behavioral plasticity, then variability in this trait is expected to depend on the social context. Hence we also compared timing patterns in different social situations, such as differences in chorus density. In chorus situations it was essential to assess the potential conditions in which some males might differentially mask more of the pulses of their neighbor than other males. Alternatively, males might attempt to minimize such overlap in the same way that has been shown in some neighboring males of *H. avivoca*. We would then expect to find predictable differences between the signals of males calling in isolation compared to those calling in larger choruses. The patterns observed in both solo calling and competitive situations will affect mating success, which we can predict from our study of female preferences (Chapter 4). Do patterns observed during vocal interactions increase or

decrease a male's attractiveness to females? Is the change in attractiveness general or do some males fare better in this regard than other males? Finally, we conducted one experiment to assess whether a single pulse could affect a male's pattern of calling or whether a train of such pulses was required. The results, in conjunction with fine-scale analyses of pair recordings and responses to the synthetic pulse train provide a tentative answer to questions about the threshold number of pulses required to alter pulse timing and whether such timing is achieved on a pulse-by-pulse basis. These results bear on mechanistic questions about signal-timing (e.g. Greenfield 1994).

METHODS

Call Characterization

We recorded the advertisement calls of 77 males of *H. femoralis* in the Apalachicola National Forest (Liberty County, FL, USA) during the summers of 2010 and 2013. Males aggregate in roadside ditches and other ephemeral bodies of water after heavy storms. For each individual, we recorded at least 120 seconds of continuous calls using a solid state Marantz digital recorder (PMD670) and a Sennheiser directional microphone (ME-66) positioned 45-60 cm from the male. We recorded the air and water temperature to the nearest 0.1°. Calls were digitized at a sampling grate of 48 kHz and analyzed using Adobe Audition v2.0 (Adobe Systems Inc., San Jose, CA, U.S.A.) or custom software (Schul, personal communication). All pulse rate data were temperature corrected to 25°C, the average air temperature during our recording sessions. We analyzed variability of call traits using R statistical software (Team, 2013).

Effects of Density and Chorus Structure on Call Structure

Males were recorded as described above in 12 choruses during the breeding seasons of 2010 and 2013. We calculated the density of each chorus by counting the number of actively calling males per square meter. After each recording, we placed flags at the original call sites (males often fled if disturbed) and measured the distance and sound pressure level of all neighbors within 2 meters of the focal male. From these data, choruses were classified as low, medium, or high density based on natural breaks in our data (small: 0.9 or fewer calling males/m^2; medium: between 1 and 1.9 males/m^2; large: 2 or more males/m^2, see Results). A single-factor ANOVA was used to compare the average within-male CV across the three density groups (Zar, 1984).

Pair Interactions

To more clearly distinguish individual male responses to nearby neighbors, we recorded 10 pairs of males calling within 2 meters of one another. We collected or silenced any surrounding males to ensure that the focal males were more likely to respond only to each other's calls. When possible, we collected baseline data of calls produced by each male when the other member of the pair was silent. Males were recorded on separate channels in a stereo file. We analyzed shifts in temporal parameters during a 2-minute calling bout in an effort to determine what type of interactions, if any, existed between calling males in a natural setting.

"Deaf Male" Experiments

Here we isolated 8 single calling males in the field in very low density choruses. After all other males were collected or silenced, we recorded baseline call data for the focal male. Then, using a single speaker connected to a 24-watt amplifier (Legacy Car Audio Inc. Brooklyn, NY, USA) and laptop computer, we broadcasted a series of 30 artificial *H. femoralis* pulses, which were regularly spaced with the average pulse period of advertisement calls, during the focal male's calling bout. The stimulus was broadcast at 85 dB at 1 meter. Because the temporal properties of the stimulus were fixed, these playback experiments were equivalent to an interaction between the focal male and a deaf rival, which obviously could not react to the calls of the focal male. To measure fine-temporal adjustment over this time period, we compared the median and standard deviation of the pulse duration and pulse period between the baseline and experimental recordings. The distribution of pulse period was not normally distributed, therefore we used a Kolmogorov-Smirnov test to compare the two call samples.

Phase Delay Experiments

Our additional fine-scale analysis of male calling behavior was derived from a phase delay experiment with eight males in the field. We interrupted each focal male's natural calling bout with a single artificial pulse, and measured the shift in his basal call period after the stimulus presentation. We repeated this procedure three times for each focal male during his calling bout. All stimuli were broadcast at 85 dB SPL at 1 meter.

RESULTS

Characterization of Gross and Fine-temporal Signal Features

The distribution of pulse periods is bimodal, with males producing pulse periods at around 80 ms and 150 ms. During continuous calling, males switch between these two peak periods, but not in a predictable fashion (Figure 3-1, Figure 3-2). Compared to the three other species with pulsed calls, this species has the highest variability in terms of pulse rate (Table 3-1). To determine if call characteristics varied with male density, we grouped males into three density classes (see methods and Fig. 3-3). A summary of the temporal and spectral characteristics measured in all three density classes is provided in (Table 3-2). As reported in Gerhardt and Huber (2002), the within-pulse structure is fairly static. The shape and period of the two to five sub-pulses were highly static within and between males; however, sub-pulse number usually varied during continuous calling (Table 3-2).

Density-Dependent Call Variation in Pulse Period

Pulse period increased significantly with density ($r^2 = 0.375$, $P = 0.005$, Fig. 3-3); however, inter-male distance and mean within-male CV of pulse period were uncorrelated ($r^2 = 0.11$, $p=0.45$). The overall lack of correlation was mainly attributable to the observation that there was no significant difference between the mean CV of males calling under low and medium densities. There was, however, a significant difference between those two groups and the high density group (Tukey HSD: small-large: $p = 0.004$, medium-large: $p = 0.001$, see Table 3-3). Thus in the densest choruses, males produce a greater

proportion of pulses in the higher range of the bimodal distribution (with pulse periods around 150 ms) than males in choruses of lower density.

Pair Interactions

To measure interactions between calling neighbors, we compared the time delays and advances of 10 focal males relative to their calling neighbor. Calls were considered synchronous if they fell within the range of +/- ½ pulse period of the focal male. Calls overlapping by more than ½ were considered alternating. The range of pulse timing varied from complete alternation to weakly synchronous, with a majority of the pulses showing some degree of overlap (Fig. 3-4). Table 3-4 shows the breakdown of the calls analyzed for 10 pairs of calling males. Within the weakly synchronous calling bouts, individual males shifted between the leading and following position. Individual males in two of the pairs (1 and 9) were matched in terms of the proportion of leading and following signals each produced; but most recordings revealed a consistent leader (Table 3-4). Individuals in four of the ten pairs showed a leadership ratio of 2:1 or greater and were considered consistent leaders. All others favored one male over the other in terms of the proportion of leading calls. Pairs also differed markedly in the proportion of calls that were alternating (Table 3-4). On average, 45% (±17%) of pulses overlapped (weakly synchronous), 0.2 % (±.03%) were produced simultaneously (perfectly synchronous), and 54.8% (±23%) were alternating (N=1,013 pulses). The degree of overlap ranged from 4.8 degrees to 351 degrees. Leading calls led by an average of 123 degrees (±96 degrees), and followers lagged by an average of 117 degrees (±91 degrees).

Deaf Male Playback Experiment

The baseline call pattern for all 8 focal males was typical of the population mean and standard deviation; however, in all experimental trials the focal male significantly adjusted his pulse period by an average of 40 ms, just 20 ms short of the rate of the deaf-male playback (Fig. 3-5, Table 3-5). Pulse period variability differed markedly between males, but the median pulse period was fairly consistent during the solo recordings (median: 82.5, standard deviation range: 2.5 – 35 ms); however, focal males clearly increased both the pulse duration and silent gap between pulses during the experiment, results in a significantly slower overall call rate (two-sided Kolmogorov-Smirnov test: $p<0.001$, Fig. 3-5). This shift also resulted in higher variability of the pulse periods compared to the solo recordings (median: 112.8, standard deviation range: 12 – 108 ms, Table 3-5). When comparing the degree of overlap between the focal male and the static recording, we found similar patterns as in the paired recording. Most of the focal males' pulses fell within the range of synchrony with the playback stimulus (+/- ½ the pulse period of the stimulus, Fig. 3-6).

Phase Delay Experiment

The response phase was fairly flat for individuals across the range of stimulus phase angles ($r^2 = -0.315$, $P = 0.1535$, Fig. 3-7), suggesting that males did not modulate pulse period on a pulse-by-pulse basis, regardless of where in the pulse period the stimulus falls. This conclusion was reinforced by an analysis of responses of males after the first pulse of a neighbor as well as in the 30-pulse entrainment test.

DISCUSSION

Pulse-rate Variability in H. femoralis

Signaling behavior is the result of an interplay between neuromuscular constraints, environmental factors, and the social context. Although the distribution of within-individual variability of different signal properties is continuous in insects and anurans, it is also distinctly bimodal (Gerhardt and Huber 2002). What is unusual about the pattern in *H. femoralis* is that the variability in pulse rate (period) is so much higher than that in most other species in which this property can be characterized as static. For example, males of the three most closely related species, *H. versicolor, H. chrysoscelis, and H. avivoca*, produce discrete calls composed of distinct pulses repeated at a species-specific rate. Pulse rate in the calls of *H. versicolor* and *H. chrysoscelis* fit the criteria for static properties because the average within-male CV is well below 4% (Gerhardt, 1991). Under solo calling conditions pulse rate in *H. avivoca* also meets this criterion, but in the presence of conspecific competitors, within-male CV is considerably higher (Martínez-Rivera and Gerhardt, 2008). Nevertheless the within-male CV of pulse rate (period) in *H. femoralis* is more than an order of magnitude higher than that of *H. avivoca* in competitive situations, due primarily to its bimodal distribution. Although there are differences among individuals of *H. femoralis*, the pulse-rate variability is consistent among males, and we did not observe individuals in which the within-male CV fell below 4.6%. One possibility is that the within-pulse (sub-pulse) structure, which is highly stereotyped, conveys species identity and thus allows for plasticity in pulse rate. Each complex pulse might thus be comparable to a call in

gray and bird-voiced treefrogs, but if so, the "call-rate" in *H. femoralis* is still far more variable than the call rate in these other treefrog species.

Fine-temporal Adjustment

In our data from calling pairs, the pulse-timing relationship ranged from weak synchrony to alternation; however, the degree of overlap for most calls was between 10% and 60%, resulting in at least partially unobstructed signals for both the leader and follower. Rarely were signals completely masked despite the high degree of variability from pulse to pulse. This pattern was also documented in a study of *H. ebraccata,* in which males mask the secondary note of conspecifics in an effort to leave their secondary note unobstructed (Wells and Schwartz, 1984b). Interestingly, these researchers found that females of this species prefer the follower (whose secondary notes were unobstructed) when presented with an overlapped set of signals.

We found an interesting contrast between the 30-pulse deaf male experiment and the single-pulse phase delay data. Whereas males can indeed adjust to the period of a calling neighbor, there was little, if any, response to a single pulse stimulus. Rather, males modulate their own signal production after experiencing a series a pulses from a neighbor. This contrast was observed in both playback experiments. Further research is needed to better estimate the threshold integration time (and the necessary number of pulses) needed for males to shift their own call pattern in response to neighbors (see below).

Role of Social Context

The effects of chorus density and activity have been investigated in other anurans and insects (Ritchie, 1992; Wagner, 1989; Bee, 2003; Bee, 2004; Schwartz, 1986; Schwartz, 1987). Several mechanisms have been identified to explain the various types of signal interactions between individuals in a chorus, especially as it relates to synchronous and alternating choruses (Greenfield, 1994). In phase delay and phase advance models, the onset of a neighboring signal resets the focal caller's internal pacemaker (e.g. rhythm generator), resulting in a shift in phase of the subsequent signal. This mutual adjustment in signal timing can result in a period of synchrony between neighbors (for example, in the synchronous waving of the crab species *Ilyoplax pusilla* (Aizawa, 1998). In contrast, the inhibitory resetting mechanism results in both the inhibition and resetting of the signaler's pacemaker (seen in synchronous choruses of *Neoconocephalus spiza,* Greenfield and Roizen (1993). Such signalers delay of the subsequent signal until after the external stimulus ceases. The relationship between callers can range from non-overlapping to almost perfect overlap (synchrony). In the case of apparent synchrony, however, individuals may be attempting to overlap the signal of their rival, usually by placing their calls in the leading position, which may give them an advantage in female attraction (Greenfield and Roizen, 1993). There is no evidence for cooperation among males in calling aggregations except in a few insects (fireflies) in which "true" synchrony occurs (Buck, 1988; Moiseff and Copeland, 2010). We found a significant increase in pulse period variability in larger choruses, suggesting that males of *H. femorali*s are influenced by the social context and

adjust their basal signaling patterns accordingly, though they are obviously not constrained by a rigid central pacemaker.

While this study does not attempt to identify all of the evolutionary processes that led to the highly variable pulse rate in *H. femoralis*, our experiments show that competition plays a significant role. Signal overlap was common in our recordings, and variability increased with chorus density. Such a pattern seems at odds with the results of playbacks to gravid females, which were most attracted by fast pulse-rate stimuli produced at a regular rate (see Chapter 4). Our observation suggests that male competition constrains the production of signals that would be expected to increase mating success. While our data did not show individual differences in maintaining fast pulse repetition rates, males did show differences in their ability to secure a leading or following position during signal interactions. If females are selective for fine-temporal signal features within the pulses, males may gain an advantage by overlapping their own signals with neighbors. Paired males often overlapped only slightly, which resulted in both individuals securing an unmasked portion of his signal in either the leading or following position.

Future Studies

In reality, several factors may influence the degree of signal plasticity in *H. femoralis*, including neighbor amplitude, time of night, energy reserves, and so forth. Considering our observation that signal variability did not correlate with inter-male-distance, it is clear that this behavior is not due simply to the location of a single nearby neighbor. A controlled experiment testing the role of these factors on signal variability would be necessary to

narrow down the major factor(s) driving plasticity. Caged male experiments in which the researcher systematically controls the distance between males would prove would address these questions more directly. Our data are limited because we only tested the ability of males to respond to a static stimulus, which is much more simplistic than the actual chorus conditions faced in nature. It will also be important to directly manipulate the degree to which males modulate pulse production in response to neighbors based on distance by testing them with stimuli that differ in amplitude. If males assess neighbors based on pulse period, we might expect stronger shifts in pulse period based solely on inter-male distance.

Individual differences in male condition may also explain differences in signal adjustment abilities. Hartbauer et al. (2012) suggest that plasticity may have evolved for energetic purposes for the tropical bushcricket, *Mecapoda elongata* and that signal variation within a calling night may be the results of a tradeoff between attempts to minimize energy expenditure and maintain synchrony with nearby neighbors. While males of *H. femoralis* do not call synchronously, differences in energy reserves may explain individual differences in the leadership abilities of males on a given night. Explicit experiments of energetic expenditure are necessary to define its potential role in calling variability for *H. femoralis.* Finally, if signal timing adjustment is under selection pressure via male competition and/or female preference, males that are better able to maintain the signal timing relationships that are most attractive to females should enjoy higher reproductive success (Richardson et al., 2008). Analysis of individual differences between males and their reproductive success are needed to test these ideas explicitly.

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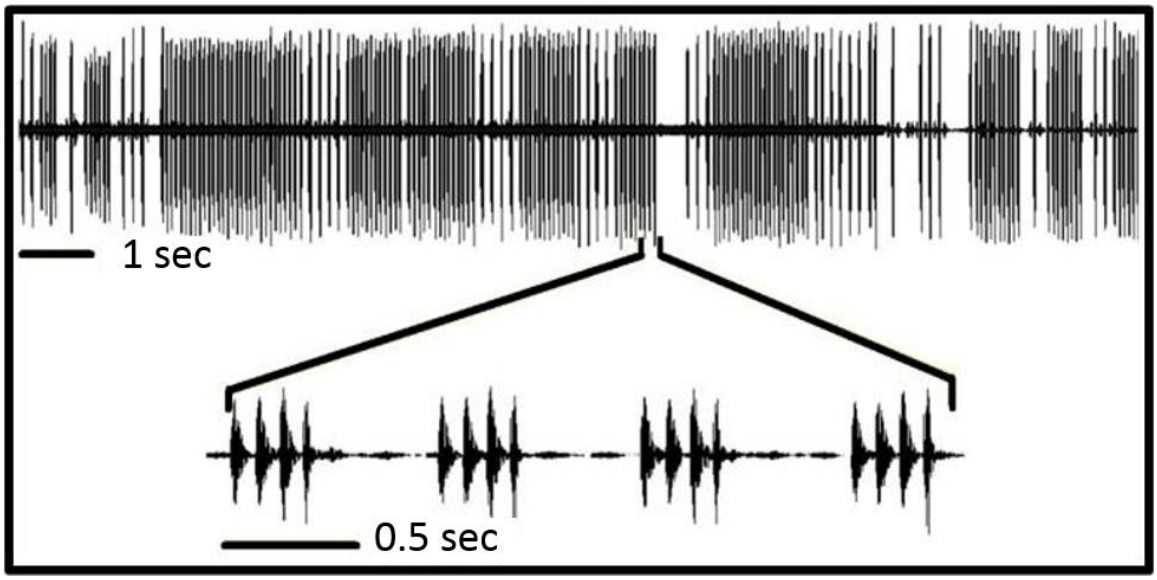


Figure 3-1. Oscillogram of typical H. femoralis

Oscillogram of a typical calling bout of *H. femoralis.* Four pulses are expanded to show the unique sub-pulse structure.

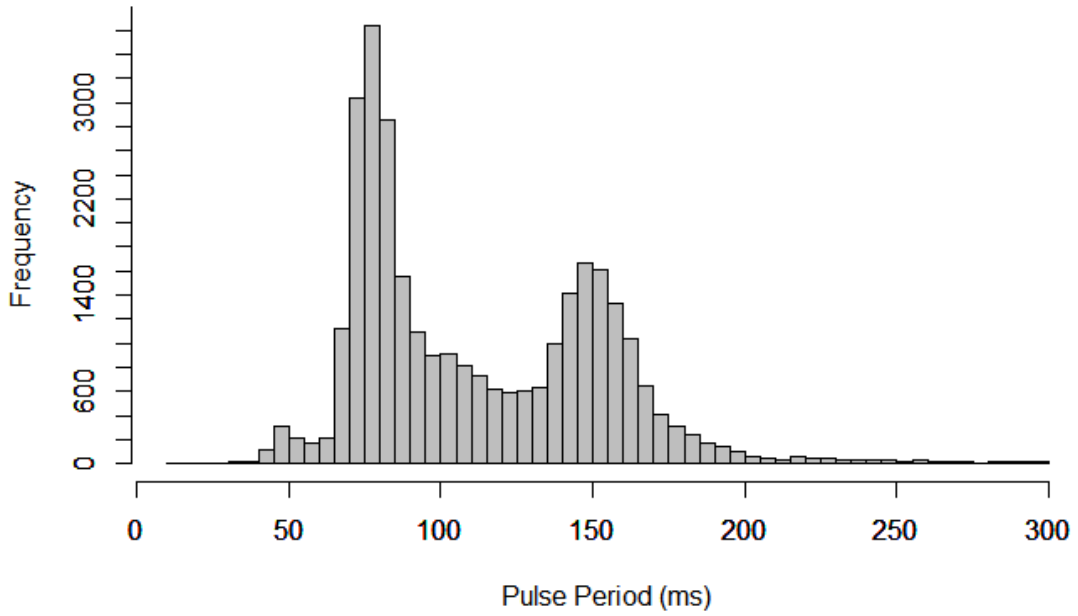


Figure 3-2. Bimodal distribution of pulse period in *H. femoralis*

Distribution of mean pulse period across 75 males. We analyzed the pulses produced by each focal male during a 120 second recording.

Species (location)	Within-male CV for pulse rate	Reference
H. femoralis (FL)	24.8% (4.6% - 93%)	this study
H. versicolor (MO)	1.5% (0.2% - 5.0%)	Gerhardt 1991
H. avivoca (LA, MS, TN)	9.0% (3.8% - 10.9%)	Martínez-Rivera and Gerhardt 2008
H. chrysoscelis (MO)	<1.0% (0.09 - 2.8%)	Gerhardt, personal communication

Table 3-1. Comparison of within-male call variability across four *Hyla* species

Pulse rate variability across four closely related North American treefrogs.

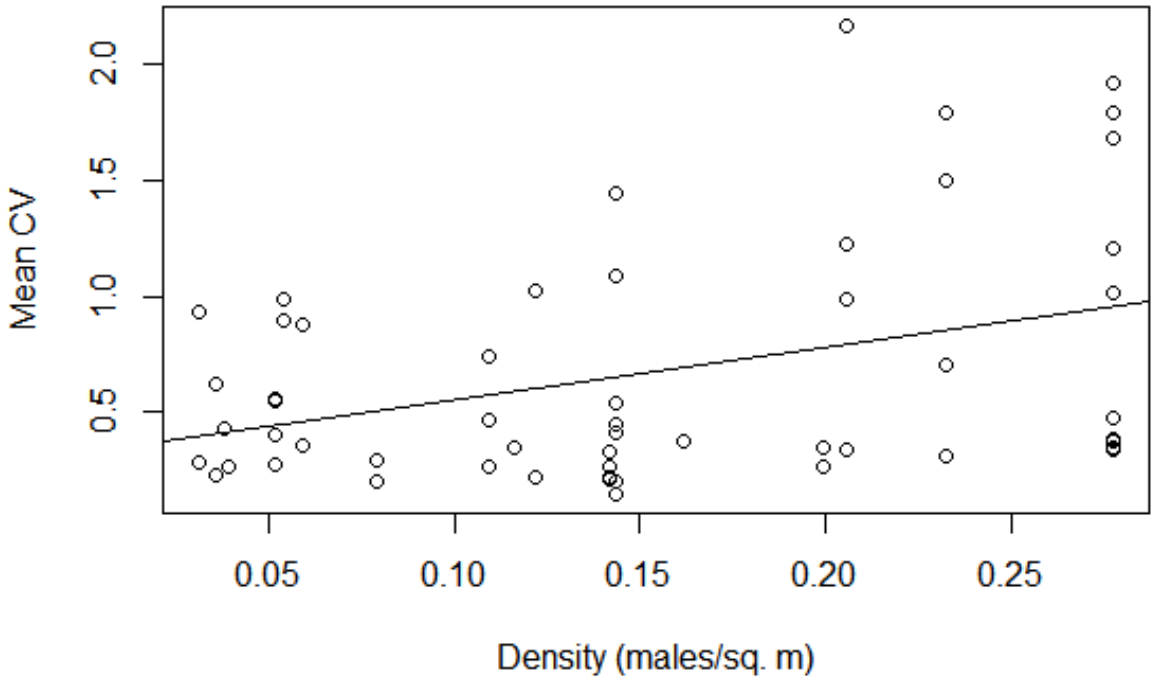


Figure 3-3. Correlation between chorus density and within-male CV

Correlation between male density and mean within-male CV for pulse period (Pulse period is the inverse of pulse rate).

	SP #	Sub-pulse Duration (ms)	Sub-pulse Period (ms)	Pulse Duration (ms)	Pulse Period	CV Pulse Period	Pulse Rate
small group (N=14)	4.23 (0.65)	4.44 (0.77)	6.38 (0.88)	35.63 (3.49)	118.06 (82.47)	0.698	9.08 (2.2)
medium group (N=20)	4.32 (0.83)	4.42 (0.78)	6.42 (0.84)	42.11 (9.23)	131.60 (57.74)	0.47	8.80 (2.9)
large group (N=20)	4.24 (0.64)	4.41 (1.02)	6.48 (1.29)	32.36 (4.60)	130.36 (62.66)	0.48	8.36 (1.3)

Table 3-2. Signal traits across density groups

Mean signal trait values (standard deviation) for *H. femoralis* across three density groups.

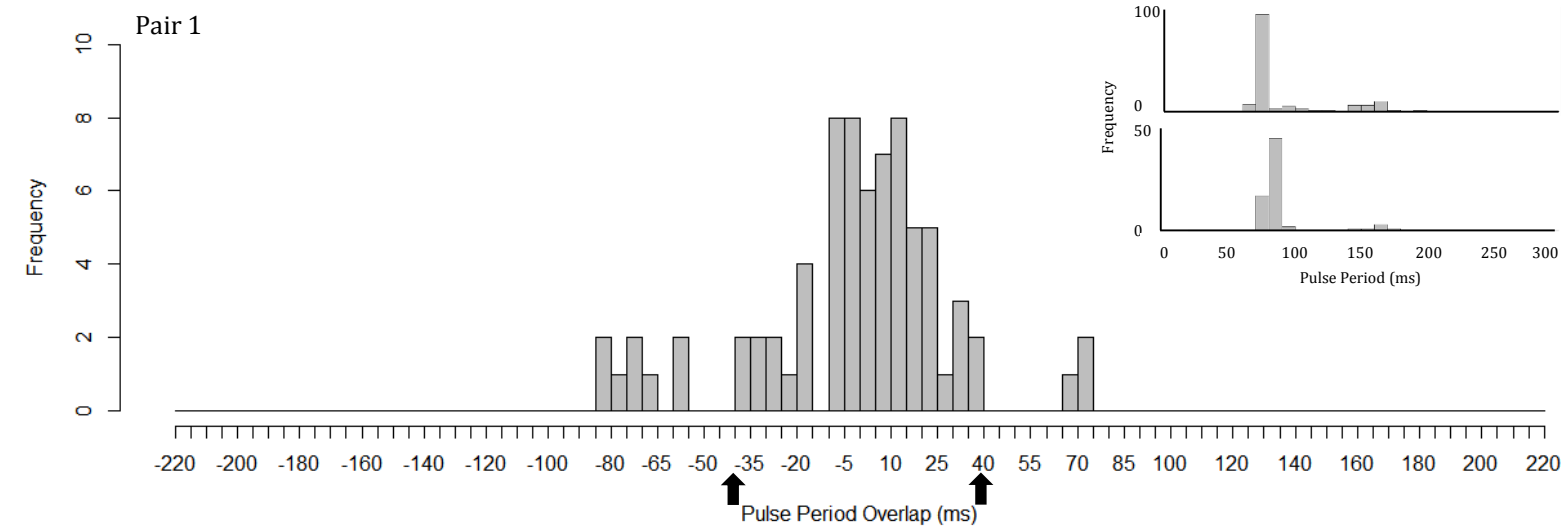
Source	d.f.	Sum of Squares	Mean Squared	F value	*p*
Density Group	2	3.587	1.7837	8.736	0.0005*
Residuals	51	10.472	0.2053		

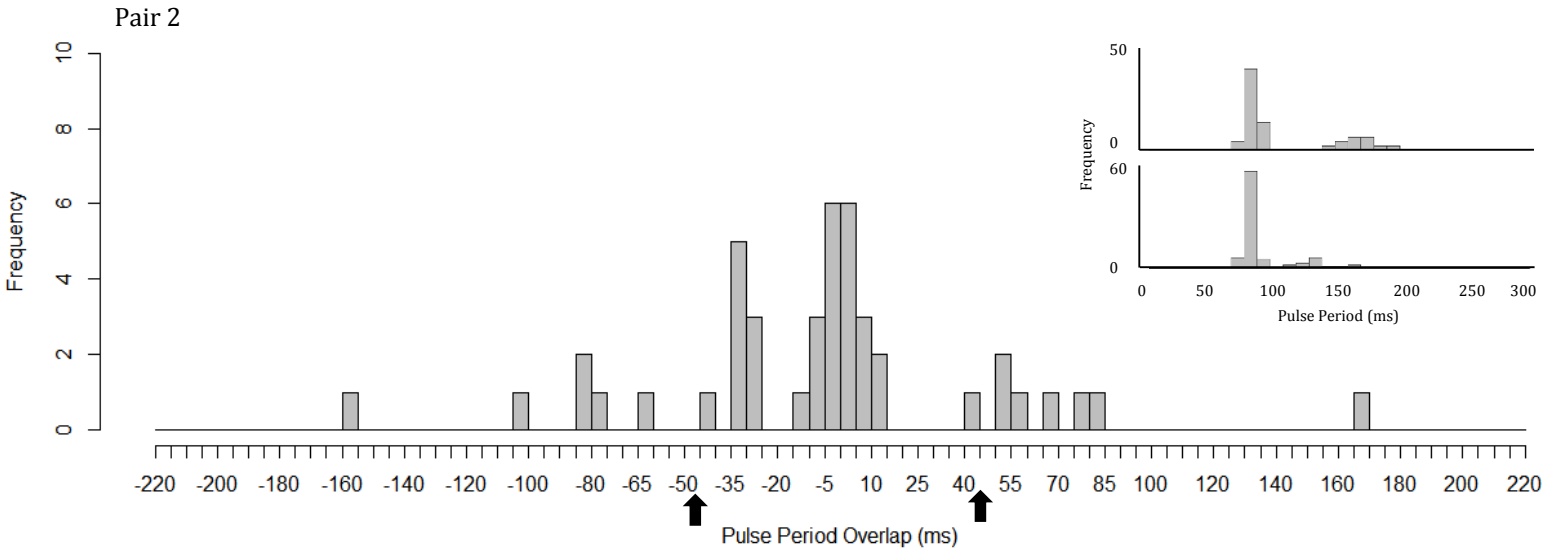
Post Hoc test: Tukey multiple comparisons of means with 95% family-wise confidence levels

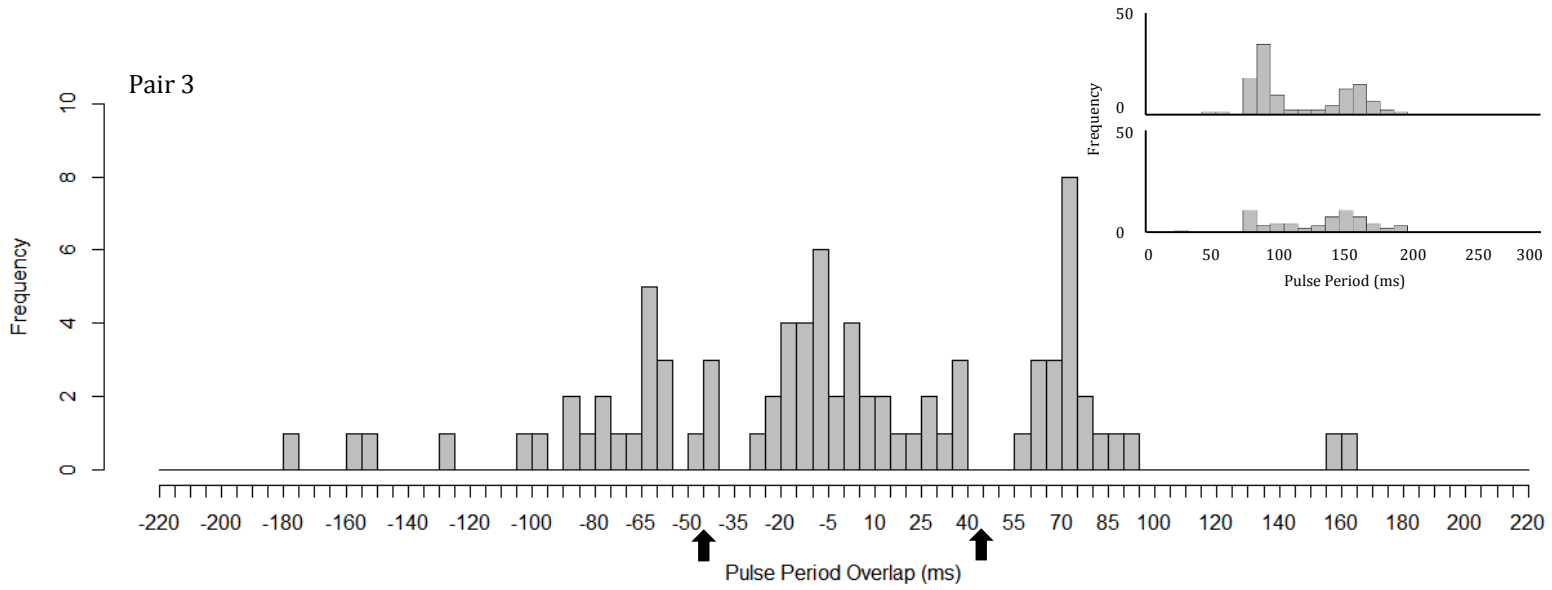
Group	Mean difference	Lower bound	Upper bound	*p*
Small-Medium	-0.072	-0.437	0.292	0.882
Small-Large	0.507	0.142	0.872	0.004*
Medium-Large	0.579	0.215	0.944	0.001*

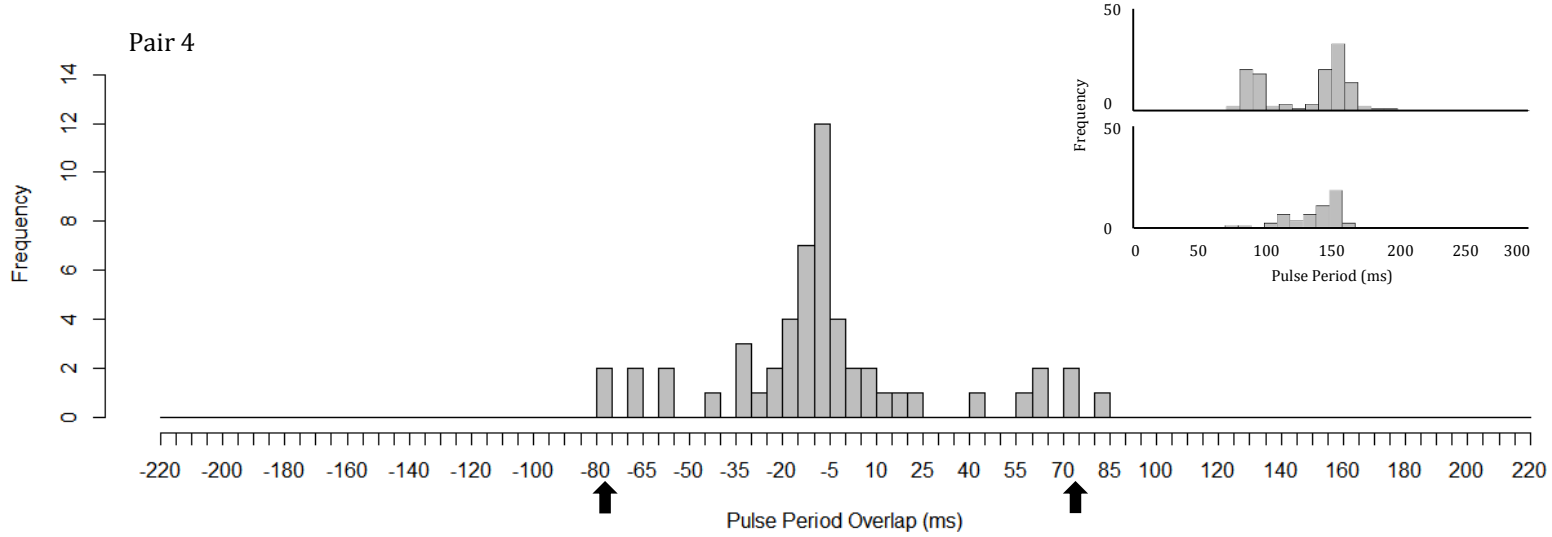
Table 3-3. Statistical analysis of call variability across density groups

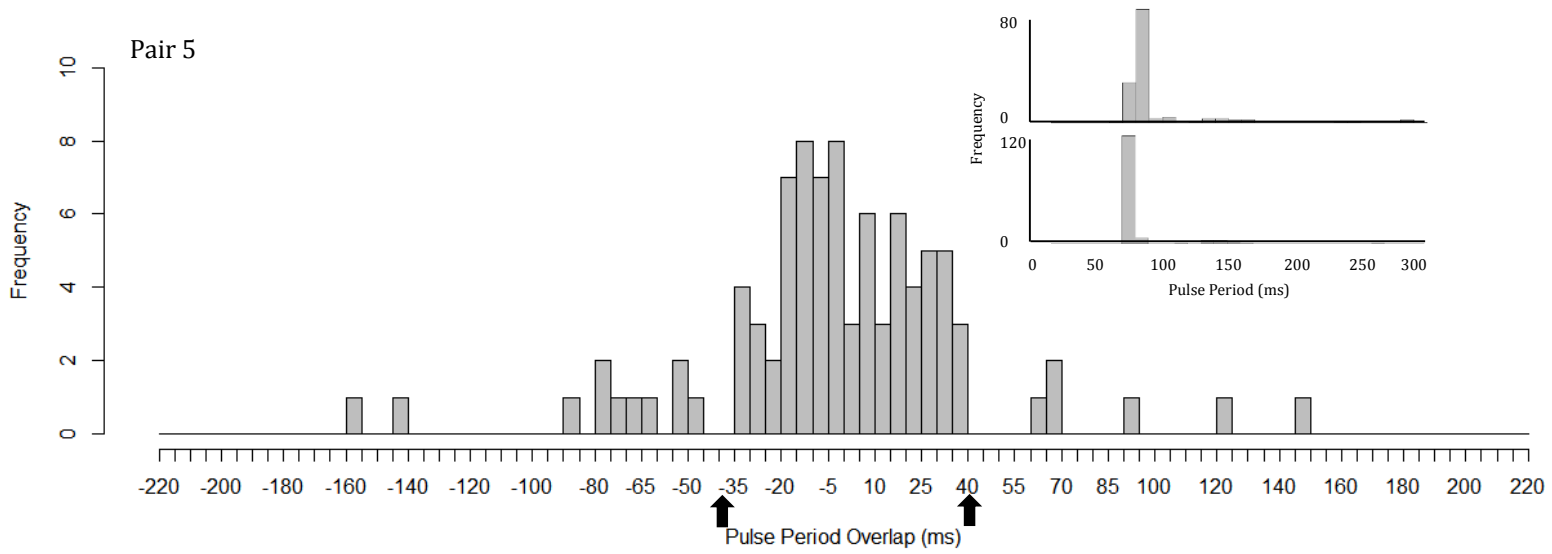
Results of ANOVA and pair-wise Tukey multiple comparisons of density group and average within-male CV for pulse period (* indicates $p < 0.05$).

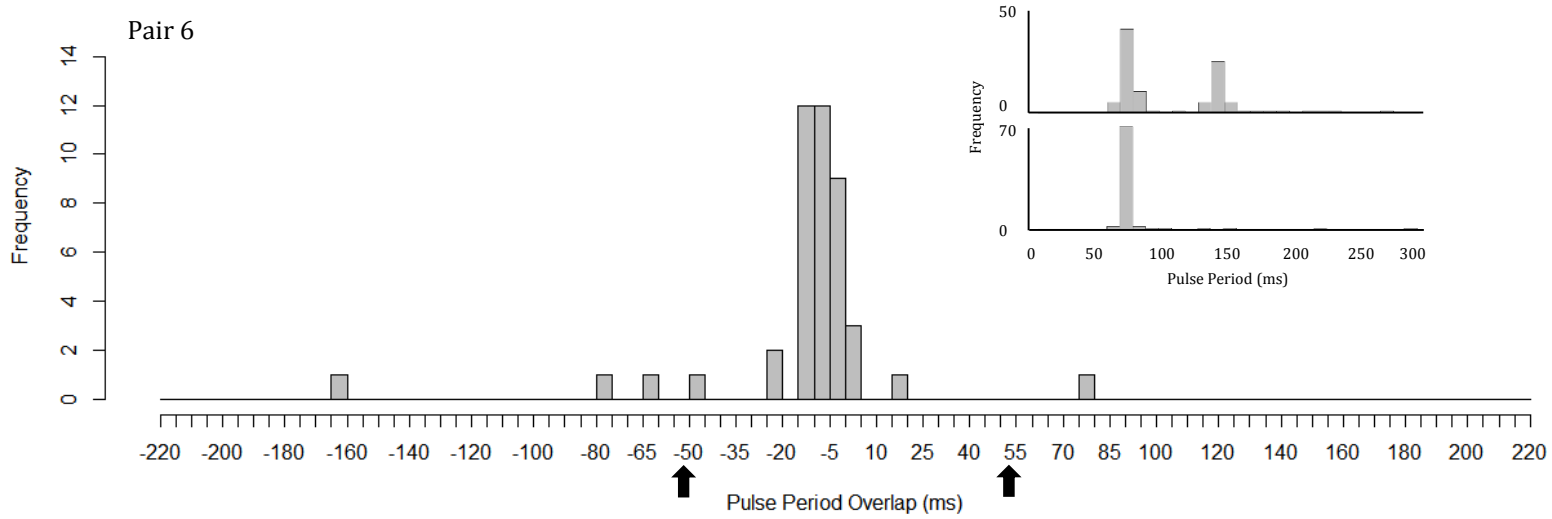

Pair 1
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
100
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)

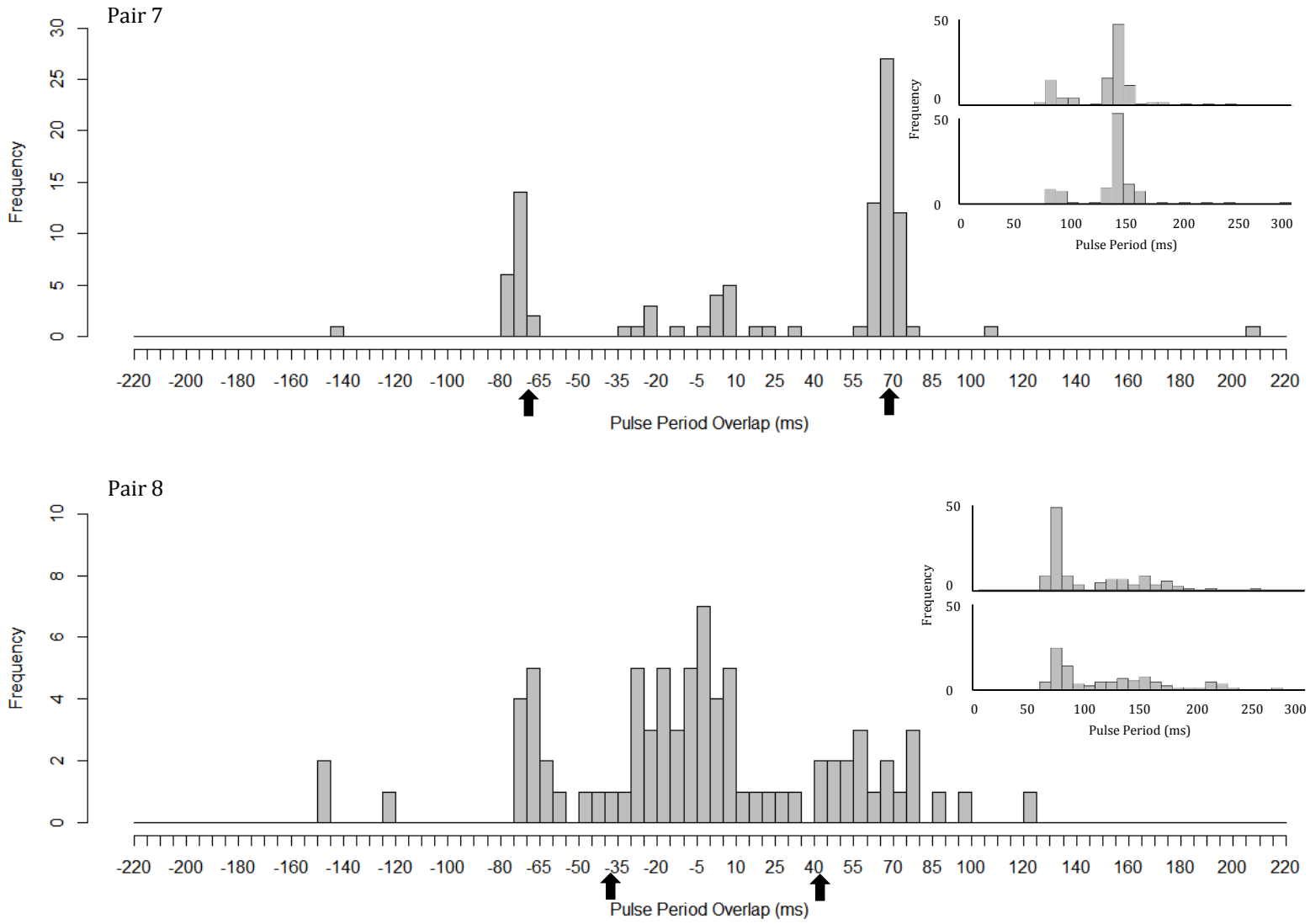

Pair 2
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
60
0
0 50 100 150 200 250 300
Pulse Period (ms)

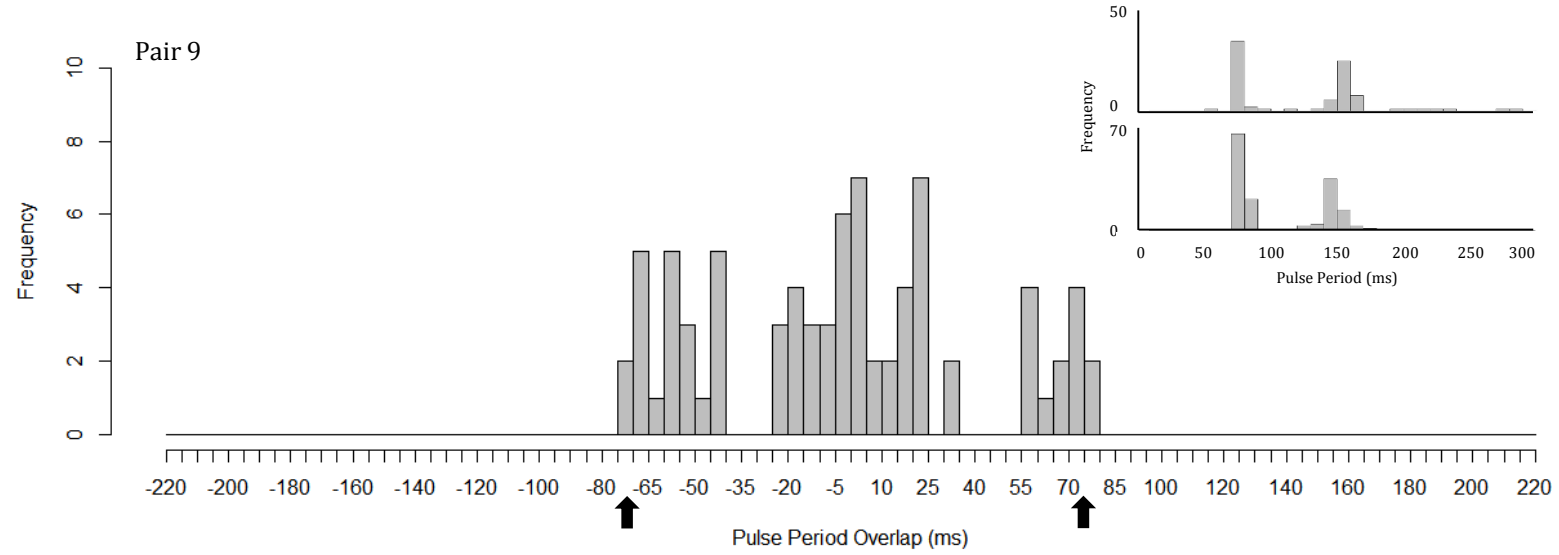

Pair 3
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)


Pair 4
Frequency
0
2
4
6
8
10
12
14
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)


Pair 5
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
80
0
120
0
0 50 100 150 200 250 300
Pulse Period (ms)


Pair 6
Frequency
0
2
4
6
8
10
12
14
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
70
0
0 50 100 150 200 250 300
Pulse Period (ms)


Pair 7
Frequency
0
5
10
15
20
25
30
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)
Pair 8
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)


Pair 9
Frequency
0
2
4
6
8
10
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
70
0
0 50 100 150 200 250 300
Pulse Period (ms)

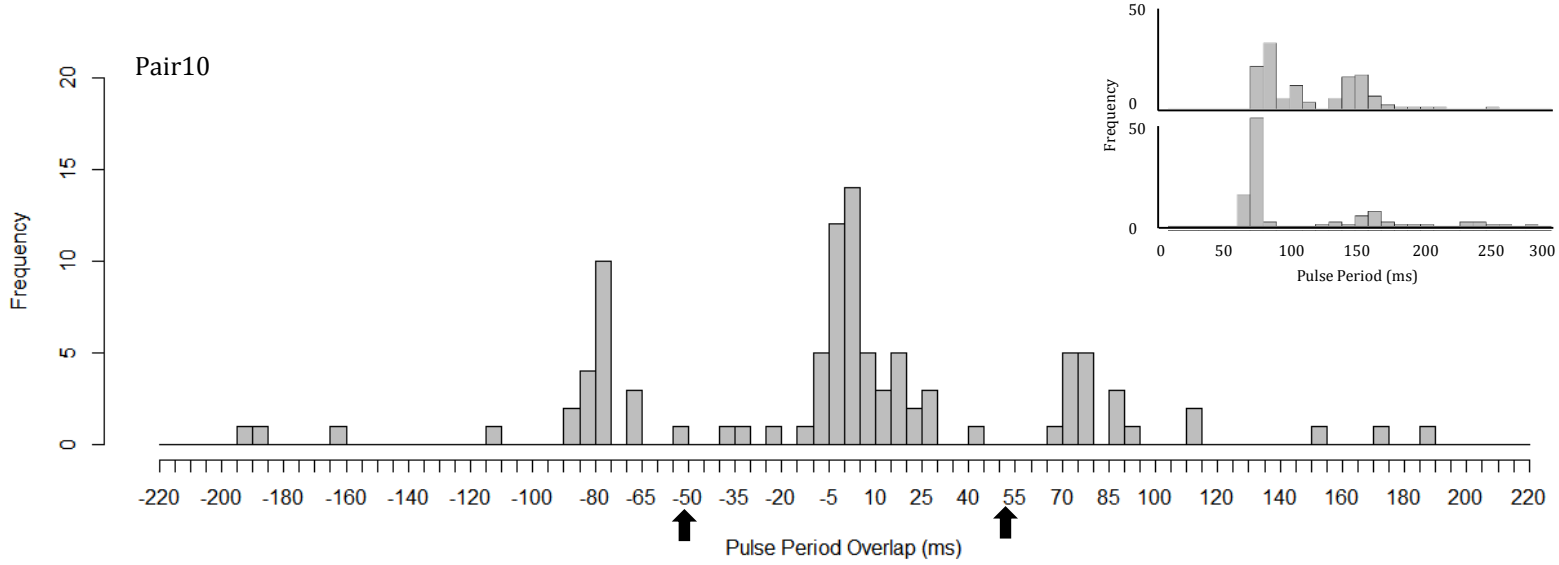

Pair10
Frequency
0
5
10
15
20
-220 -200 -180 -160 -140 -120 -100 -80 -65 -50 -35 -20 -5 10 25 40 55 70 85 100 120 140 160 180 200 220
Pulse Period Overlap (ms)
Frequency
50
0
50
0
0 50 100 150 200 250 300
Pulse Period (ms)

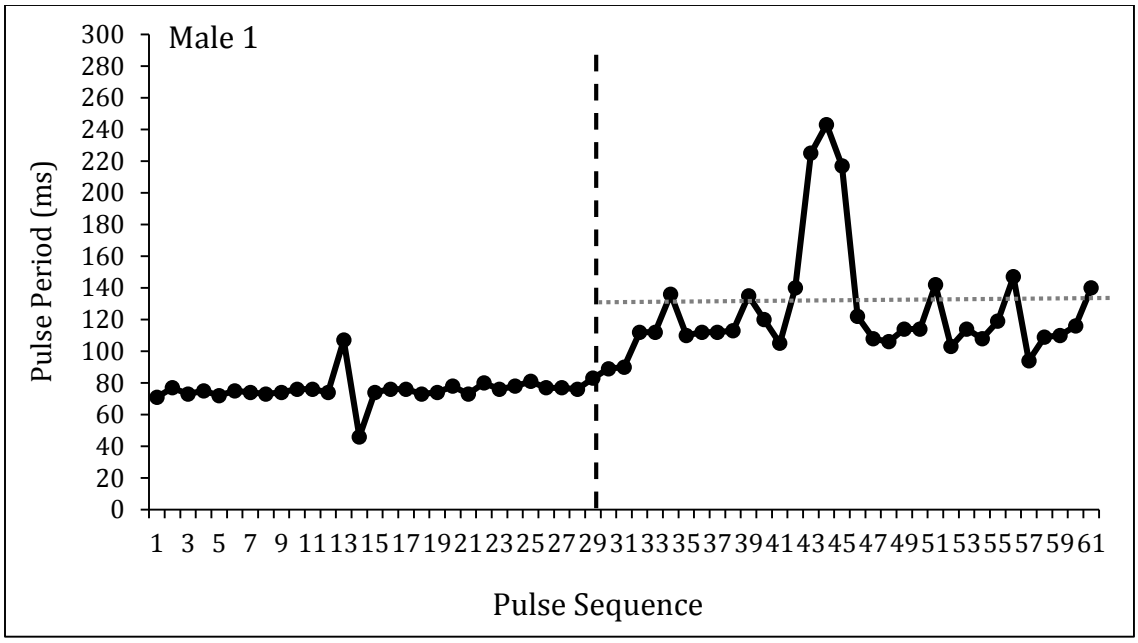
Figure 3-4. Call interactions between paired neighbors

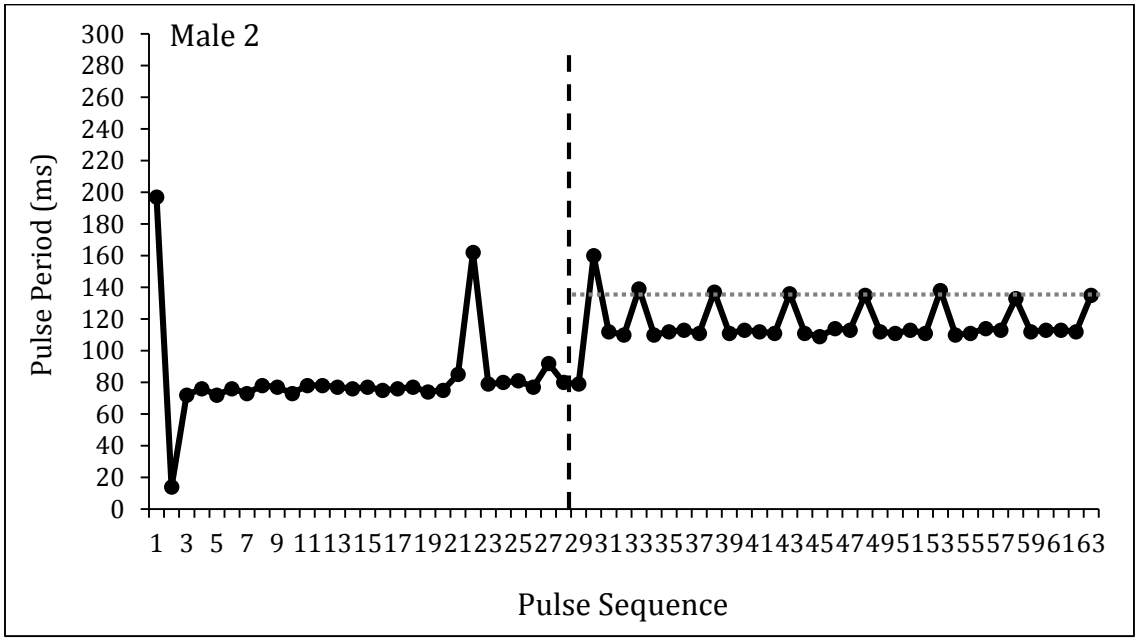
Pulse period overlap between 10 pairs of calling *H. femoralis*. Negative values indicates leading/advanced pulses and positive values indicate lagging/following pulses. Black arrows mark +/- ½ pulse period of the focal male. Overlapping calls falling within this range are considered synchronous, while peaks around the ½ mark are considered alternating. Inset: pulse period histograms of individual calling males. Top trace: focal male, bottom trace: neighbor. Note the distribution of pulse periods was similar between paired males during the recording.

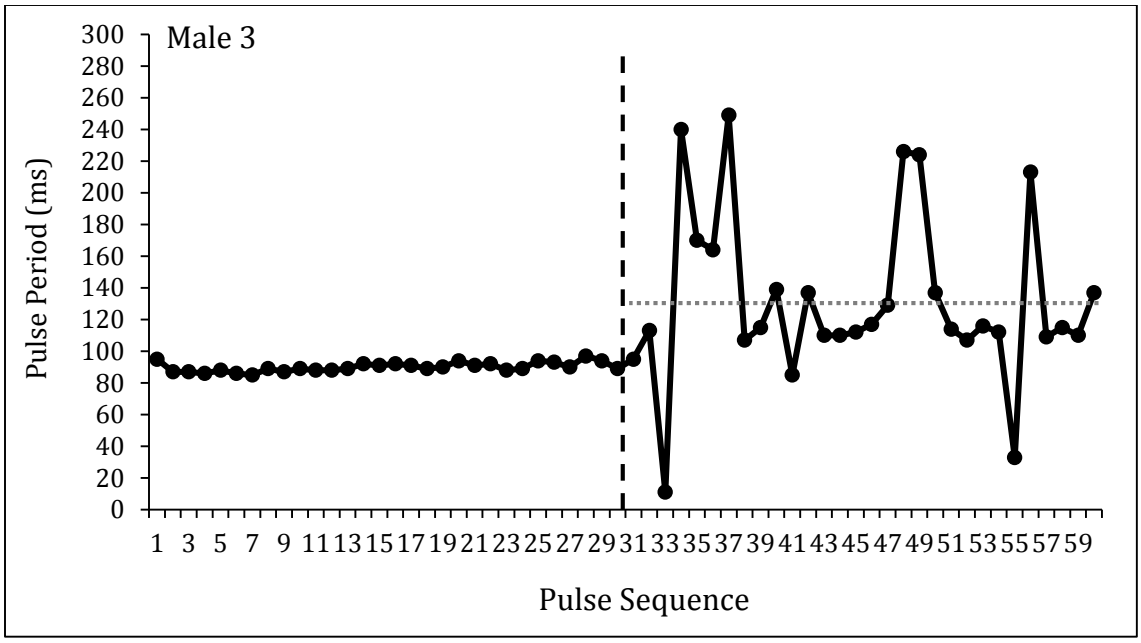
Pair	1	2	3	4	5	6	7	8	9	10
½ Median Pulse Period (ms)	41	45	46.5	75	41	52.5	71.5	42	73	51.5
Interaction Type	S	S	S & A	S	S	S	S & A	S & A	S	S & A
Leadership Interactions										
Proportion of pulses led by male A	0.39	0.22	0.19	0.52	0.32	0.36	0.06	0.26	0.27	0.16
Proportion of pulses led by male B	0.38	0.13	0.13	0.13	0.22	0.05	0.10	0.18	0.28	0.30
Proportion of alternating pulses	0.23	0.65	0.68	0.34	0.46	0.60	0.83	0.55	0.45	0.53

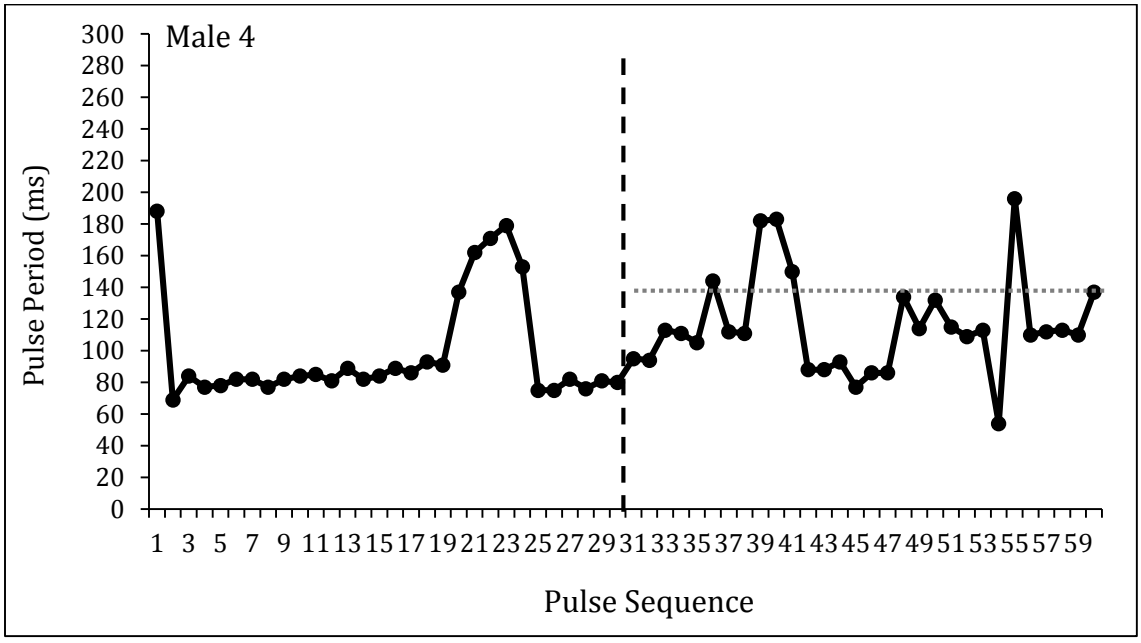
Table 3-4. Analysis of call interactions between calling pairs

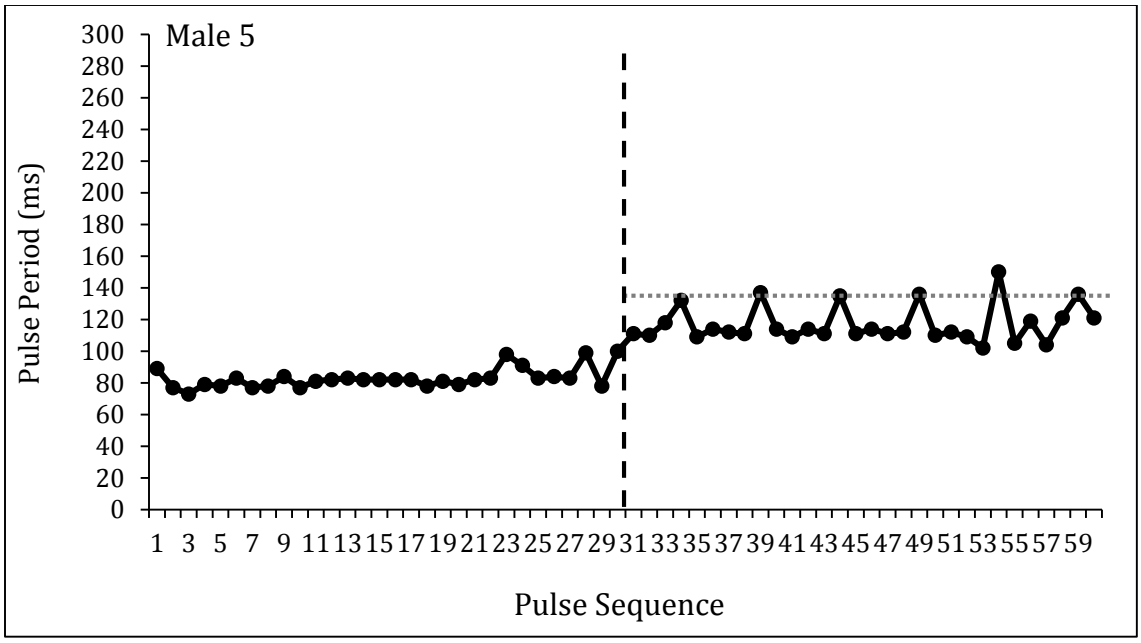
Breakdown of interactions between 10 pairs of calling males. Interaction types were classified as synchronous (S) or alternating (A) based on the distribution of overlapping pulse periods. Pulses falling within +/- ½ pulse period were considered synchronous. Most interactions were classified as weakly synchronous, however 4/10 pairs showed clear bouts of alternation. Individuals varied in the ratio of leading:following calls across most calling pairs.

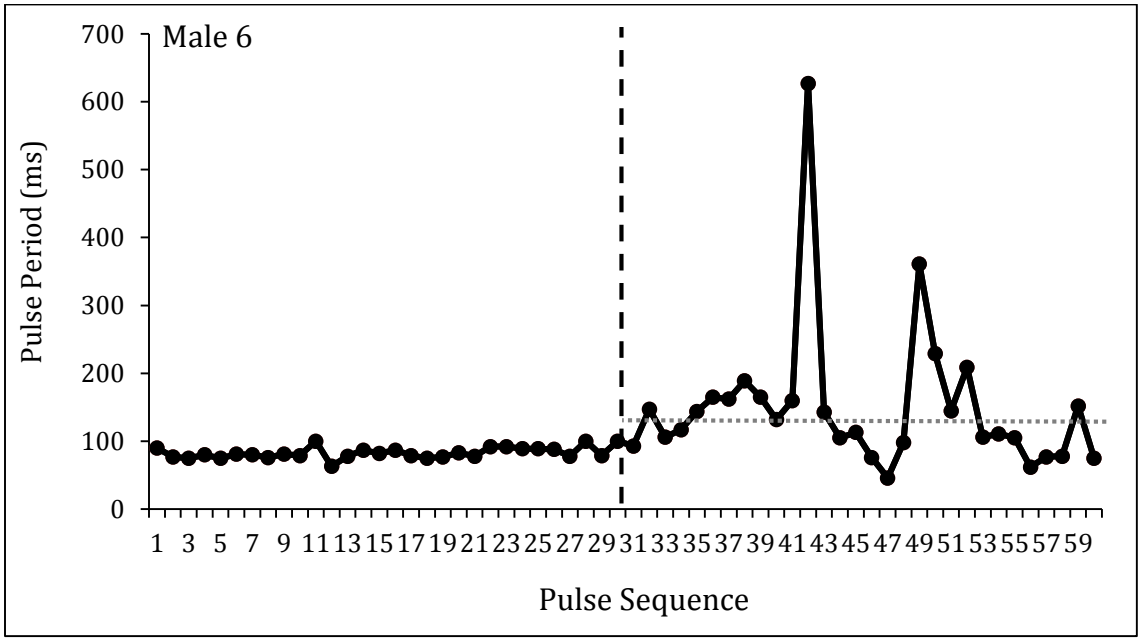

Male 1
Pulse Period (ms)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61
Pulse Sequence


Male 2
Pulse Period (ms)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63
Pulse Sequence


Male 3
Pulse Period (ms)
Pulse Sequence


Male 4
Pulse Period (ms)
Pulse Sequence


Male 5
Pulse Period (ms)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Pulse Sequence


Male 6
Pulse Period (ms)
700
600
500
400
300
200
100
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Pulse Sequence

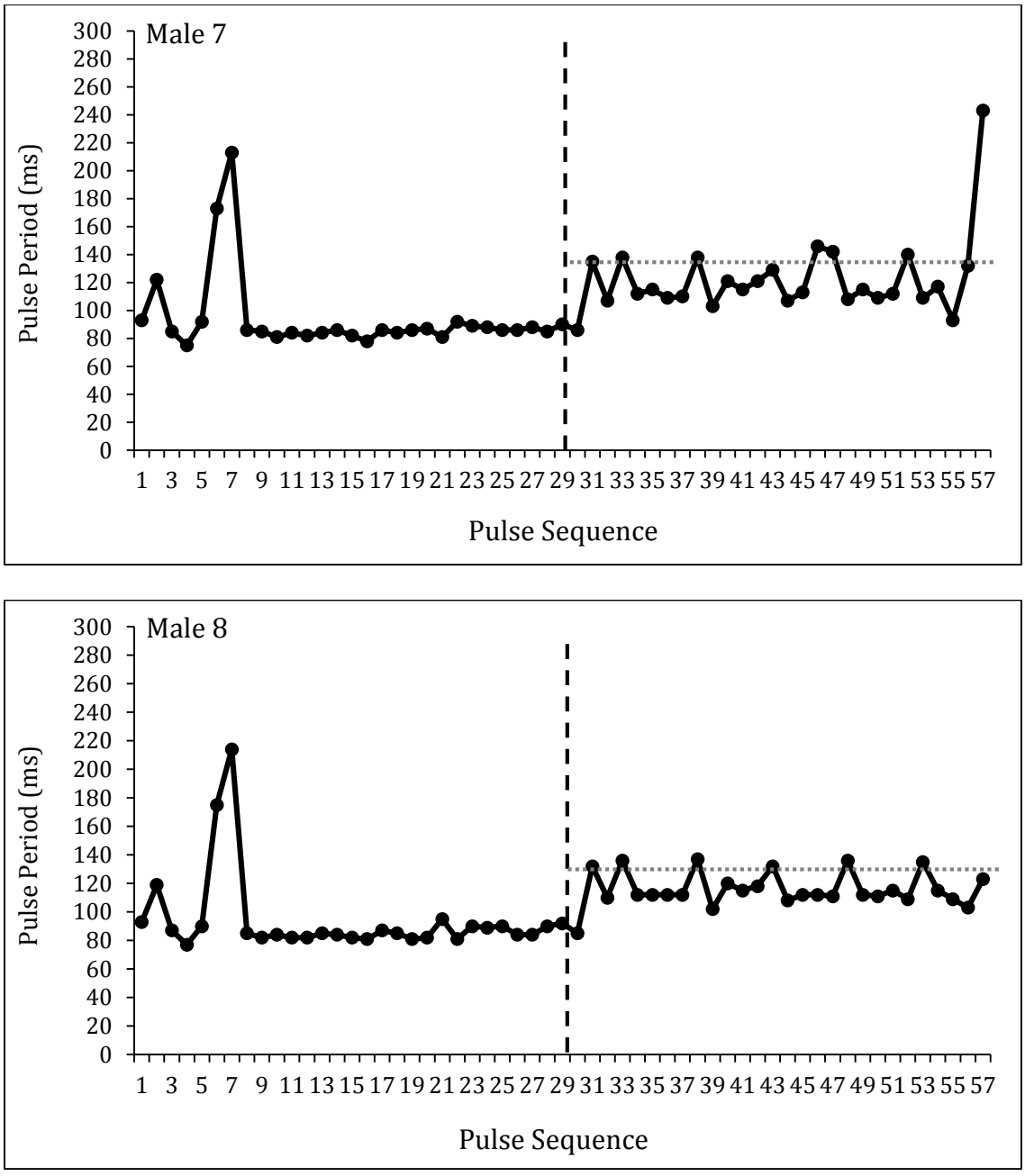


Figure 3-5. Results of "deaf male" Experiments

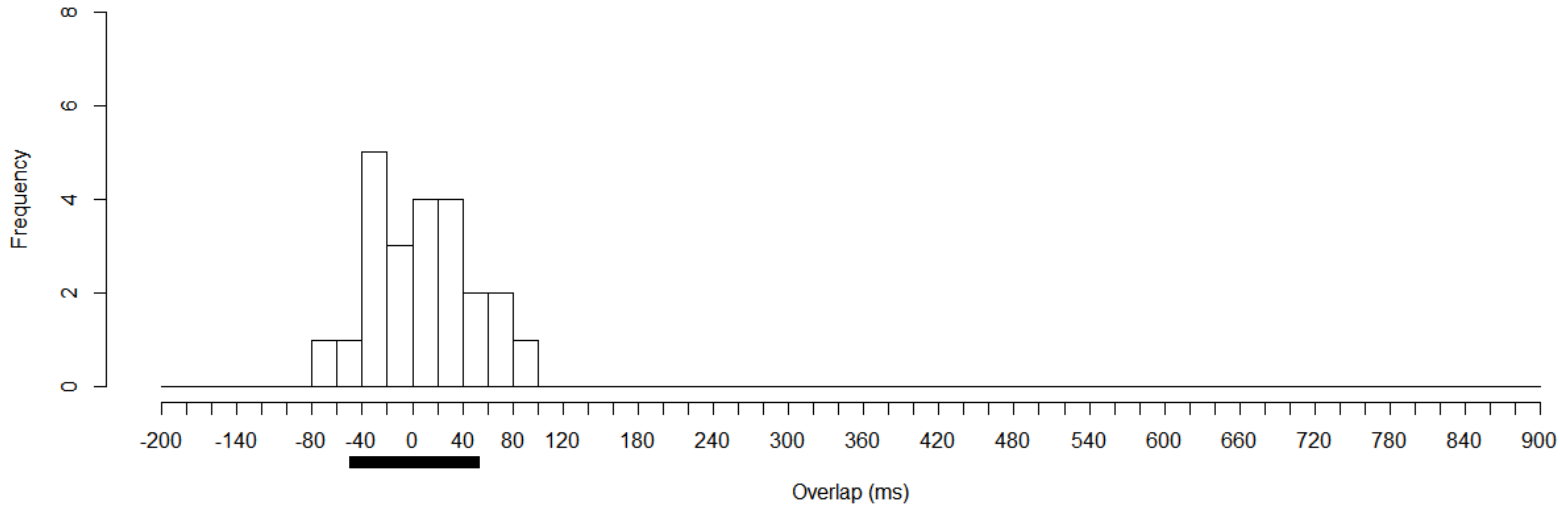
Scatterplot of focal male pulse period during the solo calling bout (prior to vertical dashed line) and deaf male experiments (after vertical dashed line). The grey dotted line represents the static pulses of the deaf male playback.

	30-pulse baseline (solo recording)				30-pulse "deaf male" trial			
Focal Male	Median Pulse Period (ms)	Standard Deviation (ms)	Median Pulse Duration (ms)	Standard Deviation (ms)	Median Pulse Period (ms)	Standard Deviation (ms)	Median Pulse Duration (ms)	Standard Deviation (ms)
1	76	8.5	29	2.8	113.5	36.4	31	3.0
2	77	30.6	29	1.6	112.0	14.0	31	2.3
3	89	2.9	34	2.4	115.0	54.3	38	6.2
4	83	35.0	32	1.8	111.5	31.5	40	5.1
5	82	6.5	31	2.0	112.0	11.6	35	4.1
6	80	8.4	32	3.7	124.5	108.7	39	6.9
7	86	29.0	34	6.1	115.0	27.9	37	5.7
8	85	29.2	34	5.8	112.0	12.0	36	3.7

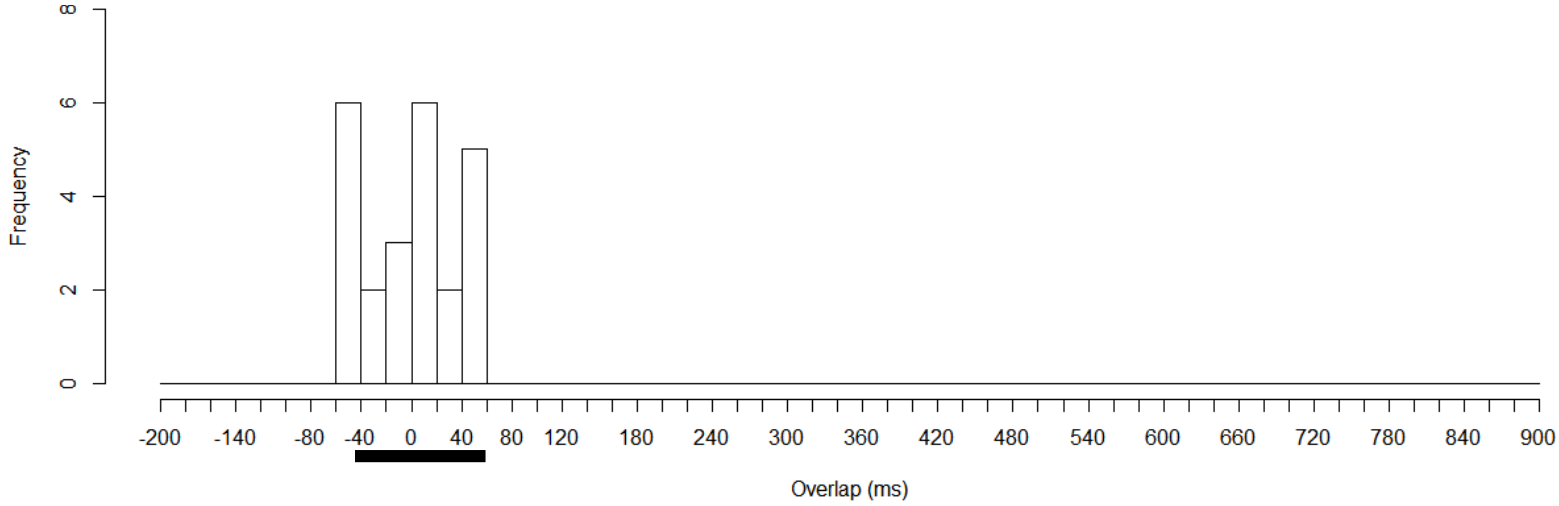
Table 3-5. Call data from "deaf male" experiments

Data from the 30-pulse solo recording and 30-pulse deaf male trial. The deaf male playback consisted of an artificial *Hyla femoralis* pulse repeated at the average pulse rate (8.9 pulses per second (pulse period = 112 ms)). For each focal male, the distribution of pulse periods during the solo recording significantly differed from the distribution of pulse periods during the playback trials ($P < 0.001$; two-sided Kolmogorov-Smirnov test). Pulse period was fairly static in the solo recording; however variability increased during the experimental trial.

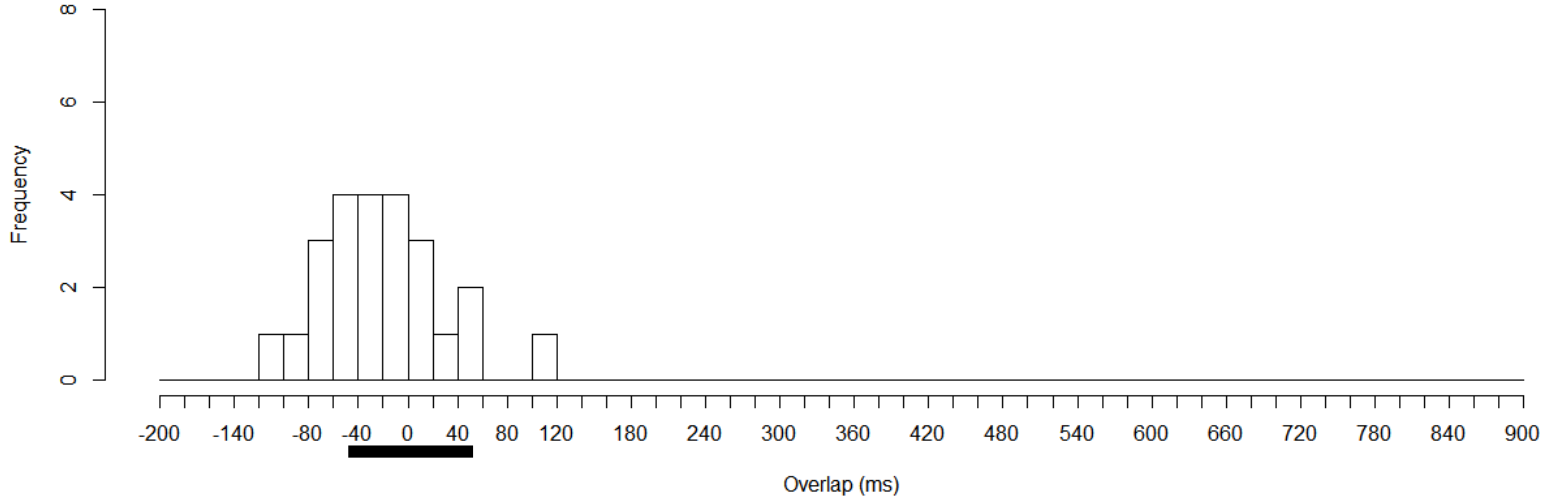
(a) Male 1



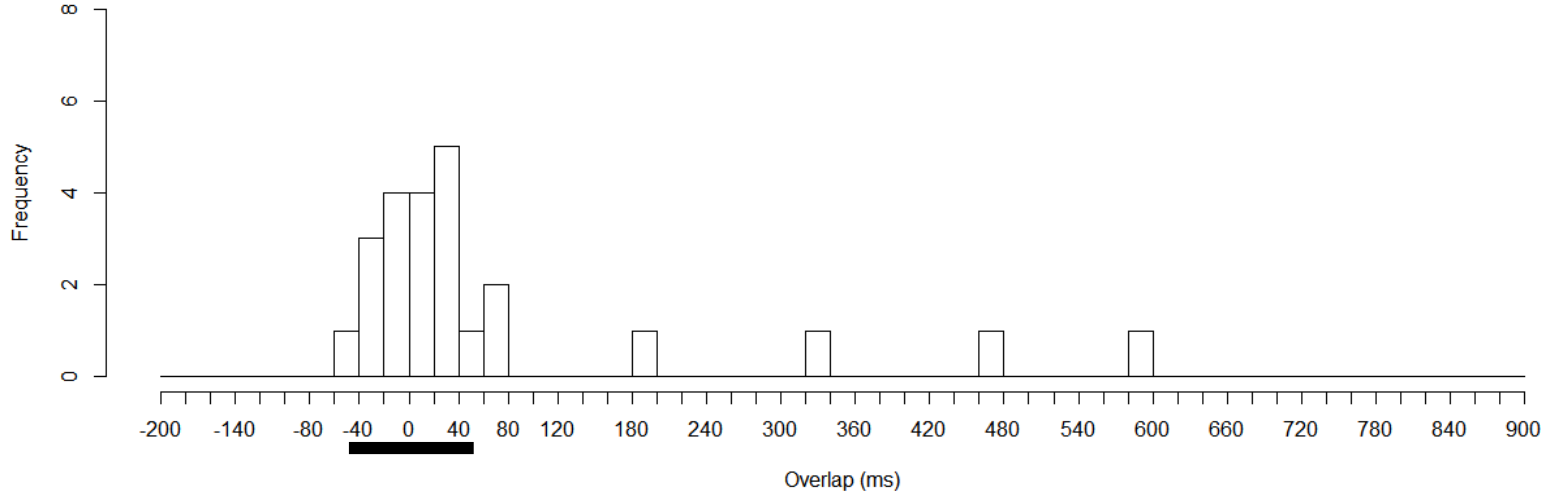
(b) Male 2



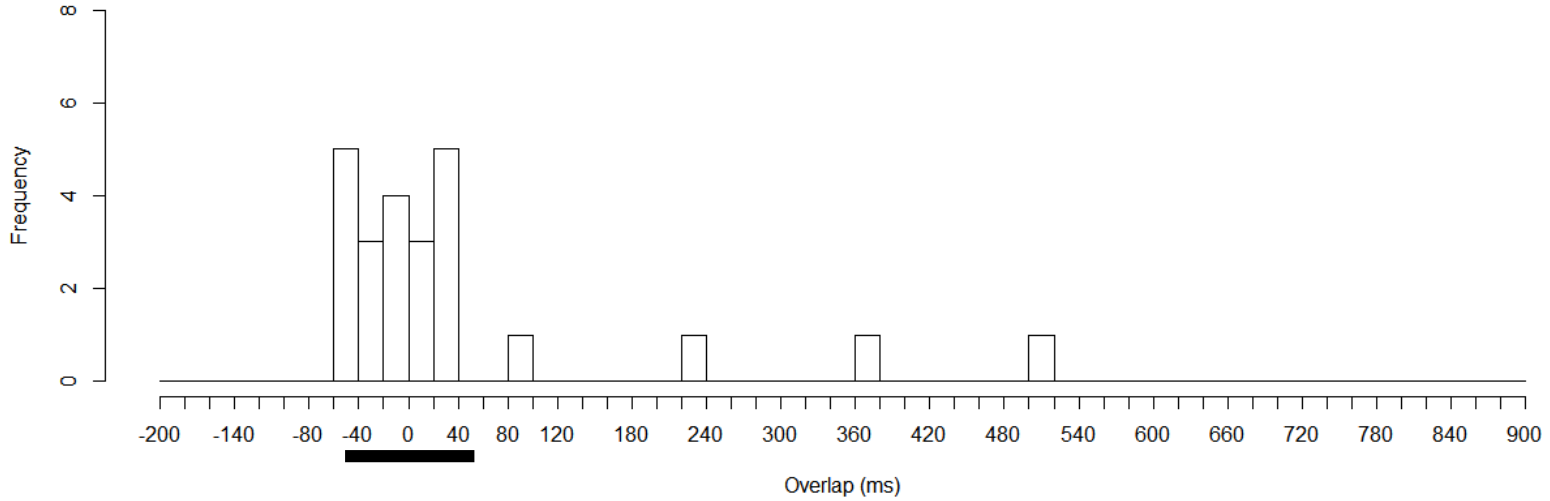
(c) Male 3



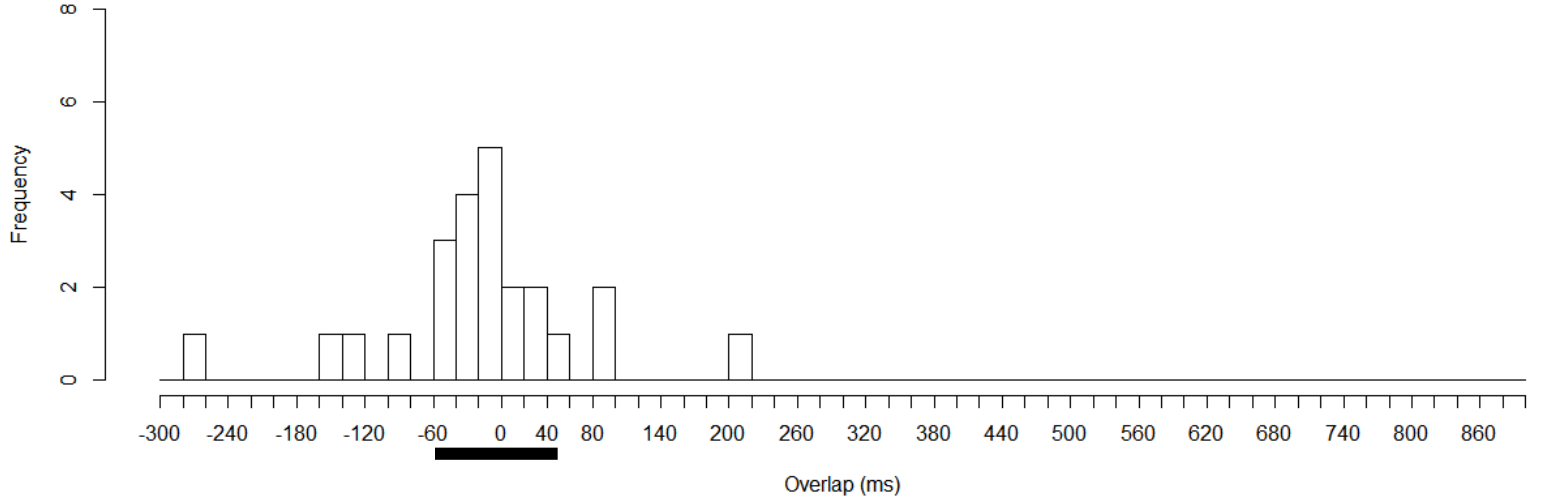
(d) Male 4



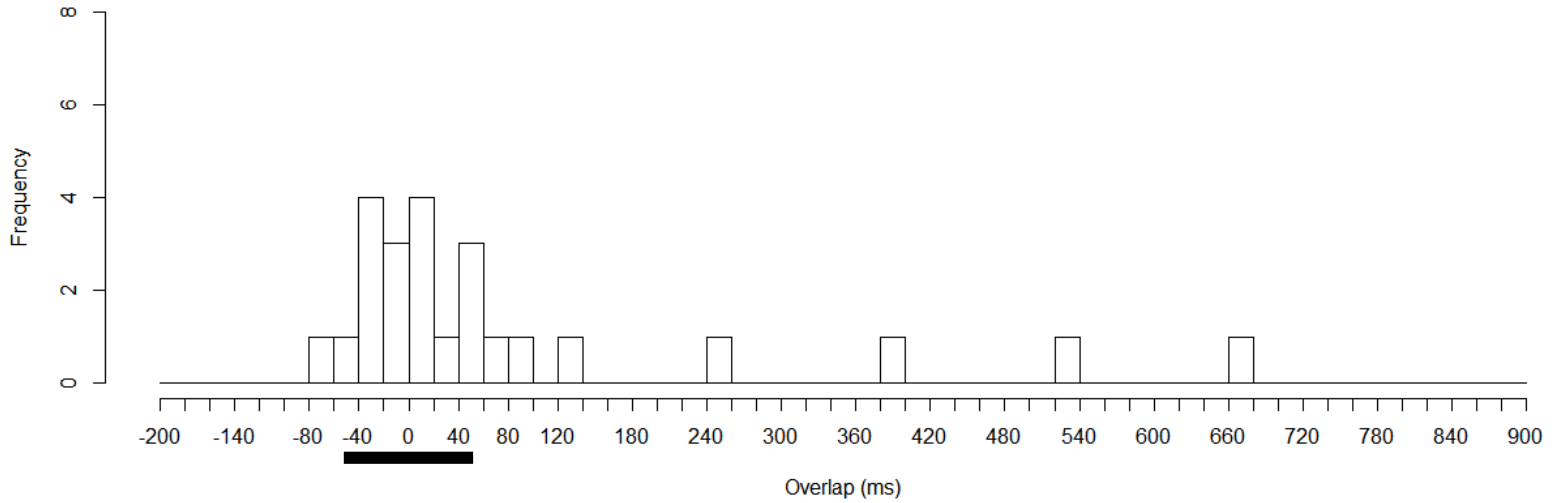
(e) Male 5



(f) Male 6



(g) Male 7



(h) Male 8

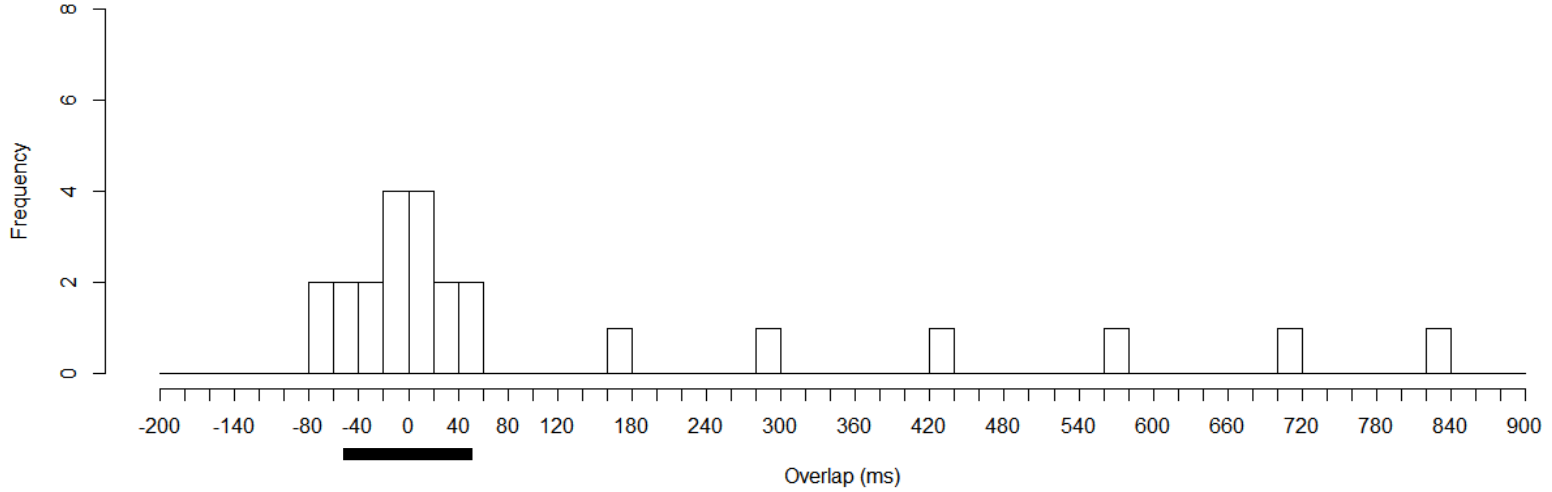


Figure 3-6 (a-h). Overlap between focal male and 30-pulse "deaf male" playback stimulus

Degree of overlap between focal male and 30-pulse deaf male stimulus. Negative values indicate the focal males' calls led the deaf male, while positive values indicate the focal male's pulses followed the playback. Pulses are considered synchronous if they overlap within +/- 56 ms (½ the length of the "deaf male" pulse period).

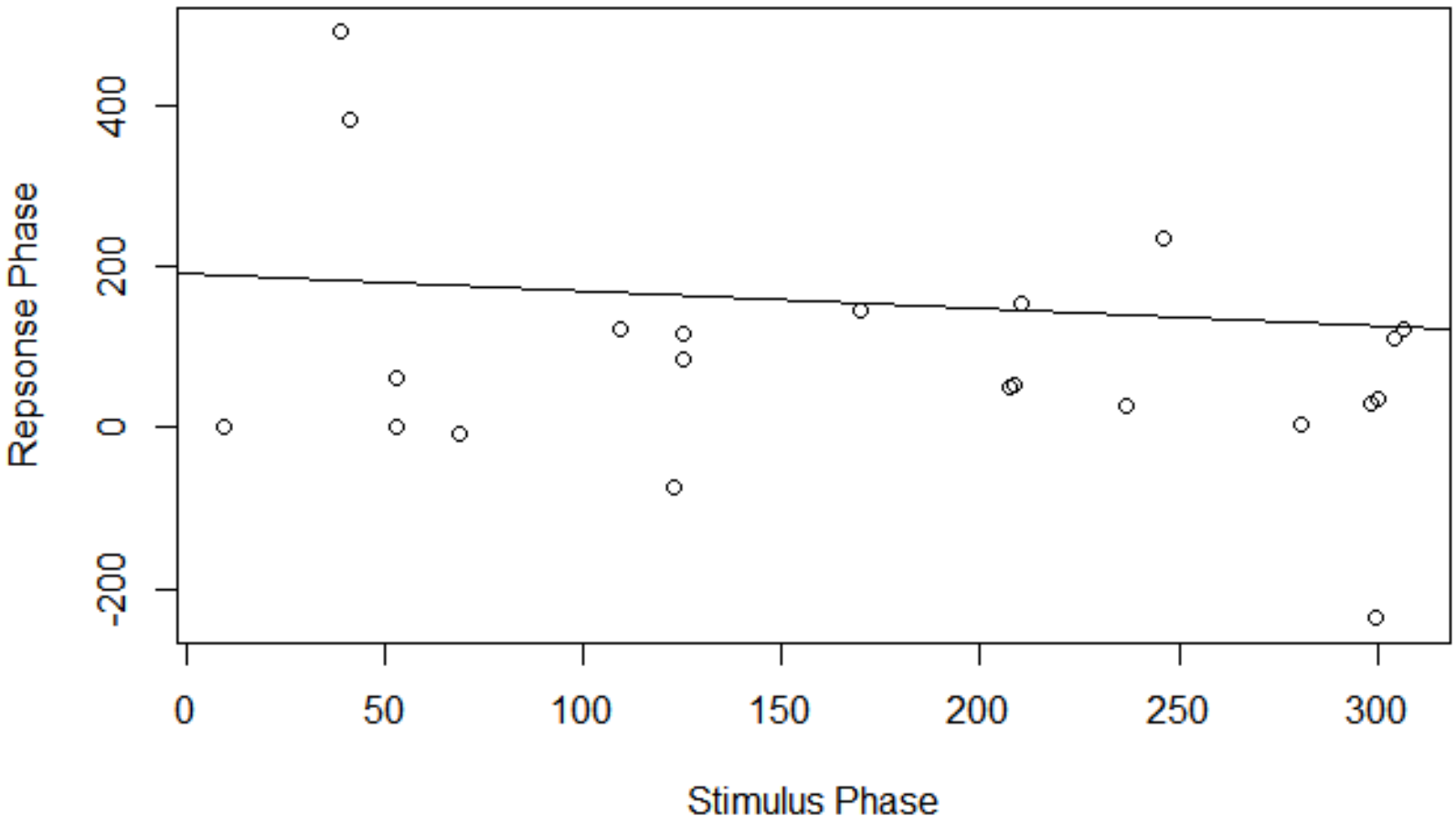


Figure 3-7. Results of phase response experiments

Focal male response phase to single-pulse stimulus trial. Males did not significantly alter response phase after being interrupted by a single pulsed stimulus (r^2 = -0.315, p = 0.153). N = 22 responses across 8 individuals.

CHAPTER 4

THE ROLE OF CALL STRUCTURE AND SIGNAL TIMING ON MATE RECOGNITION IN THE PINE WOODS TREEFROG, HYLA FEMORALIS

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INTRODUCTION

Mate choice is a strong driver of sexual signals, often exerting selective pressures that shape the morphology and behavior of the sexes (Darwin, 1871). In lekking species, the discriminating sex must often make its way through a complex dynamic environment of simultaneously signaling individuals in order to select a preferred mate. Selection is typically based on a specific characteristic or set of characteristics of the particular species, and variation among suitors may provide both qualitative and quantitative information about the quality of those individuals (Andersson, 1994; Kirkpatrick, 1987; Zuk et al., 1990; Gerhardt, 1991). Much attention has been given to the particular traits that are most important for mate choice in a wide variety of taxa across various sensory modalities. Individual variability of these traits falls on a continuum from highly static to highly variable (dynamic). Some researchers have shown a relationship between the type and strength of selection based on these traits. Notably, Gerhardt (1991) argued that dynamic properties (traits with high within-individual variation) might convey quality or condition information, and female preferences should reflect directional selection, with higher preferences for extreme values. Other researchers have corroborated this generalization in several species of insects and anurans (Welch, 1998; Howard and Young, 1998; Shaw and Herlihy, 2000). By contrast, static properties (traits with low within-individual variation) usually confer information about species identity provided that among-male variation is also low; therefore, female preferences for these traits might be expected to be stabilizing or weakly directional with preferences hovering at or near the population mean value. It is important to realize that the relationship between within-individual and among-individual

variability bears on the evolutionary consequences of selection (e.g. Arnold and Wade, 1984). If dynamic properties differ sufficiently among individuals so that they can be reliably identified, then there is the potential for an evolutionary response to directional selection. For static properties, a low among-individual variation suggests that there has been a response to stabilizing selection and such traits are suitable for promoting species recognition.

In addition to the criteria evaluated in the signals of an individual, coordination of signal production by multiple signalers may lead to higher-order signaling groups such as synchronizing or alternating choruses. Although signal synchrony could arise as a cooperative function between neighbors, most authorities view signal adjustments in the context of male-male competition (review: Greenfield 1994). Females usually prefer males whose signals lead those of a rival in time, perhaps resulting in masking interference. However, there are a few examples in which lagging signals are preferred, and still others, where females do not show preferences for leading or lagging calls (Grafe, 1996). From an ultimate standpoint, males that are able to maintain quality signal structure in the presence of high competition should have increased reproductive success. Few studies have examined these two levels of preference, and it is likely that an interaction between these two levels of preference (signal parameters and overall signal timing) will occur in species in which male density influences the level of signal overlap (Höbel 2011).

Females of the pine woods treefrog (*Hyla femoralis*), a small species found in the southeastern United States, face an additional challenge because the male call consists of pulses that are not organized in structured trills as in most other hylids in this part of the

world. Moreover, the within-male pulse rate (and its inverse, pulse period) has a highly variable, bimodal distribution within a single calling bout (see Chapter 3, Fig. 1). Previous research has shown that temporal features are important for mate recognition and preference in many *Hyla* species (Gerhardt, 1978; Gerhardt, 1982; Bush et al., 2002). In contrast to the variability in pulse rate, pulses in *H. femoralis* have a stereotyped sub-pulse structure that have a narrow range of within- and between-individual variation. Males produce pulses with an average of 4.24 (± 0.64) sub-pulses of 4.41 ms (± 1.02 ms) in duration (N= 20 males, Chapter 3, Table 2). Thus this pulse structure may be critically important for species identification and mate choice. Gerhardt (1974) demonstrated that female *H. femoralis* discriminate among potential mates based solely on mating call, and Doherty and Gerhardt (1984) showed that females also discriminated against the calls of interspecific hybrids of *H. femoralis* and *H. chrysoscelis*. Whether females based their preferences on differences in fine-scale temporal properties is unknown. Moreover, in these experiments the alternative stimuli were presented in a way that minimized their overlap and any variation in their timing relationships.

Our goal was therefore to define the preference criteria of *H. femoralis* females and then to test the strength of those preferences under different signal timing regimes. Since sub-pulse structure is highly stereotyped, we might expect this temporal structure to be under stabilizing selection by females. Given the highly plastic nature of the acoustic signal under high male density (see Chapter 3); however, the timing of neighboring signals may play an important role in female mate choice. This is first study to investigate the female

preference for temporal call features and signal timing in females of *H. femoralis.* As in other species with a relatively high call rate, we might expect females to show strong preference for leading calls or for the unmasked lagging ones (Grafe 1999). Few studies have investigated if such preferences for signal timing persist when the calls of the leader and follower differ structurally. This research addresses a significant gap in our understanding about signal plasticity as it relates to female preferences. Our research speaks to the relative strength of female preferences for signal timing, specific signal properties and their interaction and aims to enhance our understanding of signal plasticity in the dynamic social context of breeding aggregations.

METHODS

Study Subjects

Females of *H. femoralis* were collected during the summers of 2010-2013 in the Apalachicola National Forest (Liberty County, FL, USA). We collected amplexed pairs between the hours of 10 PM and 12 AM. Pairs were left in amplexus until the time of testing to maintain female receptivity. All individuals were returned to their location of capture on the following day. All experimental procedures were approved by the Animal Care and Use Committee of the University of Missouri (protocol #1910).

Playback System

Our experiments were conducted at night in a small room with blacked-out windows to eliminate as much extraneous light as possible. A single dim overhead red light allowed us to observe the females' movements. Temperatures were maintained at 25 °C ± 2

degrees, which was the average air temperature during the field season. At the start of each test, we placed the female under an acoustically transparent container in the center of a 1 meter circular arena. The arena walls were made of sheets of anechoic foam to reduce reverberations. We adjusted the playback sound level using a Larsen-Davis 800B sound level meter. The stimuli were broadcast from a personal computer to an amplifier (Legacy Car Audio Inc. Brooklyn, NY, USA) and through one or two speakers (Auvio Netherlands, Voorschoton, Netherlands), depending on the experiment. Speakers were set at 180° on the edge of the arena (0.5 meter from release location) and playback was set to 85 dB (*re* 20 µPa, fast root-mean-square, RMS). The position of the speakers was randomly changed (but still maintained at a 180° angle) between trials to prevent any directional bias.

Stimulus Design

Using custom software, we modeled a baseline pulse after the natural advertisement calls (temperature corrected to 25 °C). The average pulse contained 4 sub-pulses. Sub-pulses had a mean period of 7 ms, and the average pulse period was 121 ms, although, as stated above, variation in this trait was very high. Calls of *H. femoralis* have a frequency spectrum with most of the energy concentrated in a single, broadband peak centered at about 2 kHz. We found no difference in response rates to the artificial stimuli compared to natural exemplars. To test for preference for sub-pulse number, we modified the base stimulus by silencing the last sub-pulse period to create the 3 sub-pulse stimulus, and by adding a sub-pulse to create the 5 sub-pulse stimulus. In both experimental stimuli, the pulse period was maintained at 121 ms.

Female Preference for Sub-pulse Structure

To test for pulse structure preferences, we designed stereo files with the standard 4 sub-pulse stimulus on one channel and an experimental call (a 3 sub-pulse or 5 sub-pulse stimulus) on the second channel. The two stimuli were broadcast in an alternating fashion continuously for up to 5 minutes.

Females were tested in two-stimulus, forced-choice trials. Each individual was released after 15 seconds of playback of both stimuli. If the female moved within 5 cm of the speaker, we recorded her "choice" as well as length of time it took for her to reach the speaker. Females were subjected to one stimulus trial at a time, with a minimum 2-minute break period between these trials. We compared the proportion of females choosing the standard stimulus over the alternatives. If a female did not make a choice within 5 minutes, we score the trial as "no choice"; these data were not considered further except in estimates of the proportions of females that responded in any of these tests.

Female Preference for Signal Irregularity

We conducted a series of single-speaker experiments to test for female preference for within-male signal plasticity. Using custom software (Schul 2003), we designed several stimuli ranging from invariant pulse rates to highly irregular pulse rates. Static stimuli were created by repeating the same pulse with a fixed silent gap duration to produce a series with pulse rates of 6, 8, 10, and 12 pulses per second. Stimuli with irregular pulse rates were created by repeating the same pulse with randomly generated silent gaps between each pulse. The duration of the silent gaps were limited to ± 3 standard deviations

of the population mean silent gap. Phonotaxis towards these experimental stimuli were compared to a control stimulus, which was based on a natural recording of a local male and contained the mean values for pulse repetition rate, pulse period, sub-pulse structure, and frequency, and the natural degree of pulse period irregularity.

The trial sequence consisted of the control stimulus followed by three experimental stimuli, followed by the control, etc. until all stimuli were presented. Females were given a 2 minute time-out period between each stimulus. We presented the stimulus for 15 seconds before releasing the female. If the female moved within 5 cm of the stimulus speaker within 5 minutes, we recorded the time it took her to reach the speaker. Each female was tested on all of the experimental stimuli, unless she failed to respond to one or more of the control stimuli, in which her results were discarded. Each female was tested only once on any particular experimental stimulus (see Results for sample sizes in each test).

Female Preference for Signal Timing

To test for signal timing preference alone, we alternated two identical 4 sub-pulsed calls. In one test, we overlapped the stimuli by 50%, which resulted in masking of the first two sub-pulses of the following stimulus. In the other, we presented the following stimulus directly after the leading stimulus with no silent gap between (abutting). To test for an interaction between signal overlap and pulse structure, we designed stereo files with the standard 4 sub-pulse stimulus on one channel and an experimental call (a 3, 4, or 5 sub-pulse stimulus) on the second channel. We delayed the second channel at different

intervals to produce 100%, 50%, abutting, or alternating calls. For these experiments, we used a two-speaker, forced-choice design as in the sub-pulse structure experiments (see above). We compared the proportion of females choosing the standard stimulus over the alternatives.

Statistical Analysis

For each female who responded in the single speaker tests, we calculated the speed at which she reached the speaker by taking the reciprocal of the response time. Using the Wilcoxon signed test for paired differences, we measured shifts in the distribution of responses to each experimental stimulus relative to its paired control trial. For each experiment, we calculated the control response time as the average of the response time before and after the given experimental stimulus. Each female was run though each set of experiments, therefore; we used the Bonferroni corrected alpha to test for significant shifts in the females' responses.

We performed a two-tailed binomial test for each set of signal structure and signal timing choice tests. For the sub-pulse number experiment, the null hypothesis was that all alternatives were equally attractive. For the signal timing experiment, the null hypothesis was that the standard structure (4 sub-pulse) and alternative structure (3 or 5 sub-pulses) were equally attractive, regardless of their timing. The figures below illustrate the proportion of females choosing the standard, 4 sub-pulse signal. We present p-values for each of the tests because there we recorded only one response per female, and there is no evidence of carry-over effects in hylid frogs (e.g. Gerhardt and Doherty (1988). Because

some females responded in more than one test, we could not apply a formal statistical test to compare the results of the two tests because of the lack of strict independence.

RESULTS

Female Preference for Sub-pulse Number

In the first experiment, females were responsive in both tests and, despite a visible trend towards the longer alternative in both tests, there was no statistically significant discrimination towards any particular stimulus (two-tailed binomial test: $P>0.05$ for both tests, Fig. 4-1). Based on these results, we used the 4 sub-pulse stimulus, which is close to the mean for the population, as the standard for the remaining phonotaxis experiments.

Female Preference for Static and Irregular Pulse Rates

Females varied widely in their response to the experimental stimuli. Most approached the 8, 10, and 12 pulses-per-second stimuli at similar speeds as to the control. Response to the 6 pulses-per-second stimulus was markedly slower, however the difference was not statistically significant. In fact, there was no significant different between the distribution of female responses to any of the static stimuli and the control (Wilcoxon signed test for paired differences, $p > 0.007$ (Bonferroni corrected p-value), Fig. 4-2, Table 4-1). Thus, static pulse rates were equally as effective at eliciting positive phonotaxis as the control. On the other hand, females show significantly slower responses to the three irregular stimuli compared to the control (Wilcoxon signed test for paired differences, $p < 0.007$ (Bonferroni corrected p-value), Fig. 4-2, Table 4-1)

Female Preference for Signal Timing/Sub-pulse Structure Interaction

The results of the choice tests for leadership and sub-pulse number are shown in Fig. 4-3 and Table 4-2. In the tests for signal timing preference alone, females were equally attracted to the leading and following stimulus in both the overlapping and abutting positions (two-sided t-test 50% overlap: $p = 0.28$, alternating: $p = 1$).

In the 4 sub-pulse versus 3 sub-pulse test, most females chose the shorter stimulus in the following position when it abutted the leading stimulus (binomial t-test $p<0.05$, Table 4-2). There was no significant preference for either stimulus when the two overlapped by 50% or 100%, or when the stimuli alternated. In the 4 sub-pulse versus 5 sub-pulse test, females again significantly preferred the following stimuli, which was the longer-duration alternative, when the alternatives overlapped by 50% and when the following stimulus abutted the leading (binomial t-test, $p<0.05$ for both tests, Table 4-2). There was no significant preference for either stimulus when the two overlapped 100%, which resulted in complete masking of the 4 sub-pulse stimulus. To test for the effect of signal overlap alone, we tested two 4 sub-pulsed stimuli at 50% overlap, alternating, and abutting positions. Females did not show significant preference for the leading or following stimulus in this trial (binomial t-test, $p>0.05$ for all three tests, Table 4-2).

DISCUSSION

Female Preference for Fine-temporal Properties – Evidence of Weak Selective Pressure?

Our results show that pulse rate is an important criterion for female mate recognition in *H. femoralis*. Despite high within-male variability of this trait, females were

equally responsive to static pulse rates within the natural range of variation. Highly irregular calls (beyond the natural "bimodal" distribution) were significantly less attractive to females. Thus, females of *H. femoralis* showed no selectivity for irregularity outright, but preferred signals that fell within the typical range of variability. This result runs counter to a study of the drosophilid fly (*Zaprionus mascariensis*) in which males produced and females preferred irregular/arrhythmic signals (Bennet-Clark and Leroy, 1978).

The high degree of variability in pulse rate of the male advertisement signal represents an interesting departure from the behavior of close relatives, and, combined with the results of this study, suggests that females exert only weak selective pressure on the gross patterning of temporal traits of conspecific males. This contrasts with the stabilizing preferences for pulse rate shown by female Cope's gray treefrog *H. chrysoscelis* (Schul and Bush 2002; Gerhardt 2005).

The weakly directional or stabilizing preferences for pulse duration (as tested in terms of sub-pulse number) in *H. femoralis* may represent parallels with preferences in eastern gray treefrogs which require a minimum pulse duration and then respond very well to a wide variety of synthetic stimuli with different pulse rates and durations (Schul and Bush 2002). Playback experiments with females of the bird-voiced treefrog also suggest that there is a minimum effective pulse duration and that females tolerate a wider range of pulse rates than do Cope's gray treefrog (Martinez-Rivera and Gerhardt, 2008). We note that pulse duration in the calls of *H. femoralis* is comparable to that in the calls of *H. avivoca.*

Considering the structure of the calls of these closely related species, it may be more appropriate to reframe the definitions of the signal feature of *H. femoralis* to more closely align with those of the other pulsed callers in the clade. By considering the pulses and sub-pulses of *H. femoralis* as calls and pulses, respectively, it is less surprising to find higher within-male variation and evidence of directional selection on call rate by females. Furthermore, if we consider the static nature of the sub-pulse structure in *H. femoralis* to be analogous to the relatively stereotyped pulse structure and rates within these other species, our results indicating weak directional selection reflect the general trend among these species. An investigation involving a greater range of sub-pulse alternatives is needed to further clarify the strength of preference for this trait. The modest increase in phonotaxis towards calls with more sub-pulses may simply reflect a general preference for increased sound stimulation.

Further experiments are also needed to tease apart the specific recognition mechanism (e.g. duty cycle, absolute signal duration, etc.). Discrimination is limited to the fine-temporal resolution of the female's auditory neurons so that sub-pulses produced at such a rapid rate (approximately 125 sub-pulses per second) might be unresolvable by the female auditory system. One example is the lack of a preference in females of *Neoconocephalus robustus,* for an unpulsed synthetic model and the model with the 200 pulses/s amplitude-modulation of the natural call of conspecific males (Deily and Schul, 2004). These authors speculated that the auditory system may still be able to extract information regarding the presence or absence of a silent interval, and in any event,

differences in fine-scale temporal properties are always reflected in spectral differences, which may be useful for females.

Female Preference for Signal Timing

Calling density varies greatly across spatial and temporal scales during the breeding season; hence we tested the fine-temporal criteria in the context of a dynamic social environment in which signals often overlap, creating a range of phase delays between signals. Our data suggest that signal timing may play a strong role in female mate choice. Females showed strong preferences to the follower when its call abutted that of a leader (0% overlap) or was overlapped by 50% in both the 4 vs 3 and 4 vs 5 tests, but not in the 4 vs 4 tests. These results may suggest that there may be a threshold lag period which elicits positive phonotaxis to the following stimulus. The results of a study by Grafe (1999) provide similar behavioral results in running frogs (*Kassina Kuvangensis*). While investigating leader preference in females, female running frogs chose the following call when the calls overlapped by between 10 and 25% (Grafe, 1999). Our results may also suggest that preference for the following position may be encouraged by short phase delays, but the lack of significant results in the 4- vs. 4 sub-pulse tests complicates these findings. It is possible that preference for the following signal is minimized when competitors are matched, which may explain the lack of preference in the 4 sub-pulse standard test. Experiments testing the more fine-scaled degrees of overlap (50% and below) and other experiments using matched sub-pulsed stimuli would lend further support to these claims.

Neurophysiological studies may provide support for the role of short delays as a mediator of follower preferences. For example, in a study of the tropical katydid (*Mecopoda elongata),* Siegert et al (2011) tested the bilateral responses of TN1 neurons at various leader-follower time delays. They showed that when the delay between leader and follower is small (less than 20 ms), the neuron on the side corresponding to the following signal fired more strongly than the neuron on the leading side. Once the delay increased, the effect was reversed and preference shifted to the leading signal. Their study thus shows the potential of the auditory system to respond preferentially to a following stimulus. Behavioral studies in this and other species are needed to determine if these neurophysiological findings apply to behavioral interactions in nature. Furthermore, specific behavioral tests in *H. femoralis* and others showing this behavior are needed to test the strength of the follower preferences. For instance, mate choice may be reversed to favor the leading caller if the following stimulus is presented at a lower amplitude relative to the leading stimulus.

Leader preferences are the most commonly reported result of studies of signal timing preferences, and two main perceptual processes have been proposed to explain its prevalence. The precedence effect occurs when the receiver perceives two sound sources arriving from different locations as a single sound, and orients to the source of the leading sound. This effect has been demonstrated in humans and across a variety of taxa (humans: Zurek, 1987; birds: Dent and Dooling, 2004; insects: Wyttenbach and Hoy, 1993; frogs: Marshall and Gerhardt, 2010). Marshall and Gerhardt (2010) provided support for the precedence effect in females of *H. versicolor* by demonstrating female preference for calls

with a leading pulse, even if the call with leading pulses began after the first call of the alternative with lagging pulses. Females of *H. versicolor* are highly selective for pulse duration, and it is clear from their study that females orient towards the source of leading pulses, not simply the first sound that is perceived (Marshall and Gerhardt, 2010).

If, as suggested in our study, preference for the follower is indeed a strong driver of mate choice in *H. femoralis*, it is unlikely the precedence effect plays a strong role in the perception of females. In all experiments involving signal overlap, females never showed a significant preference for the leading stimulus, regardless of the degree to which it led the alternative, suggesting that females do not orient to the first-heard appropriate sound. The other proposed process, physical masking, may be a more appropriate explanation for our current results. If, on the one hand, females are highly selective for sub-pulse structure, we might expect leader/follower preferences to shift in experiments in which the alternatives differ in fine-temporal structure (e.g. a standard pulse containing sub-pulses versus an unmodulated pulse with no sub-pulses). On the other hand, if preferences are strictly based on the orientation to the following stimulus when alternatives overlap, females might be expected to prefer an otherwise less attractive stimulus simply because it occurred in the following position. Physical masking was cited as potential mechanism for follower preference in the Neotropical frog *H. ebraccata*. Males produce a two-part call and attempt to mask the secondary note of their neighbor, leaving their own secondary note exposed to females. Females preferred the follower in this instance (Wollerman, 1999). If physical masking is occurring in *H. femoralis*, we might expect males to adjust their signal timing in order to expose (avoid masking by the leading pulse of their competitor) their final sub-

pulses during overlap. Although our analyses of natural recordings of paired males in the field show active modulation by males during signal interactions, it is unclear if this behavior is due to selective pressure to avoid masking.

Masking may also explain the failure of females to discriminate when alternative signals occur simultaneously. When we presented alternating or completely overlapping stimuli (regardless of sub-pulse number), females did not show a preference for either alternative. Thus, the window of opportunity to increase phonotactic attractiveness relative to a competitor arises when calls partially overlap. Results presented in Chapter 3 suggest that males do indeed make fine-temporal adjustments within the acceptable range of overlap determined by our female preference tests.

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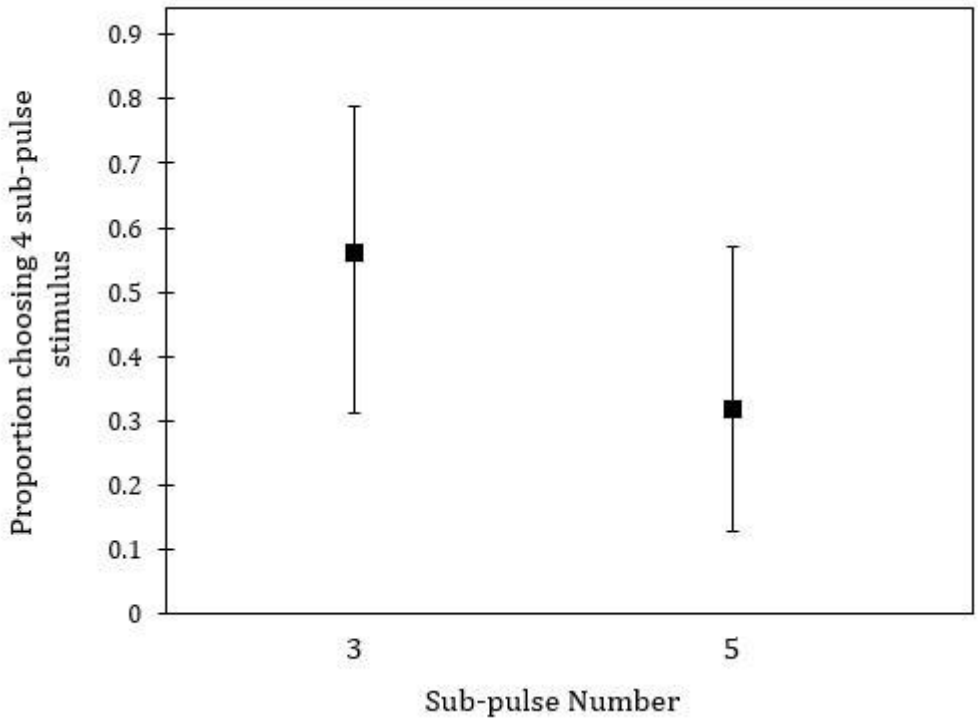

Figure 4-1. Female preference for sub-pulse number

Results of sub-pulse structure experiments. Females did not significantly discriminate between stimuli based on sub-pulse number. For the 3 sub-pulse vs. 4 sub-pulse test, 56% chose the mean value (CI: 0.31 - 0.79, N=18). For the 4 sub-pulse vs. 5 sub-pulse test, 32% chose the mean value (CI: 0.13 - 0.57, N=19).

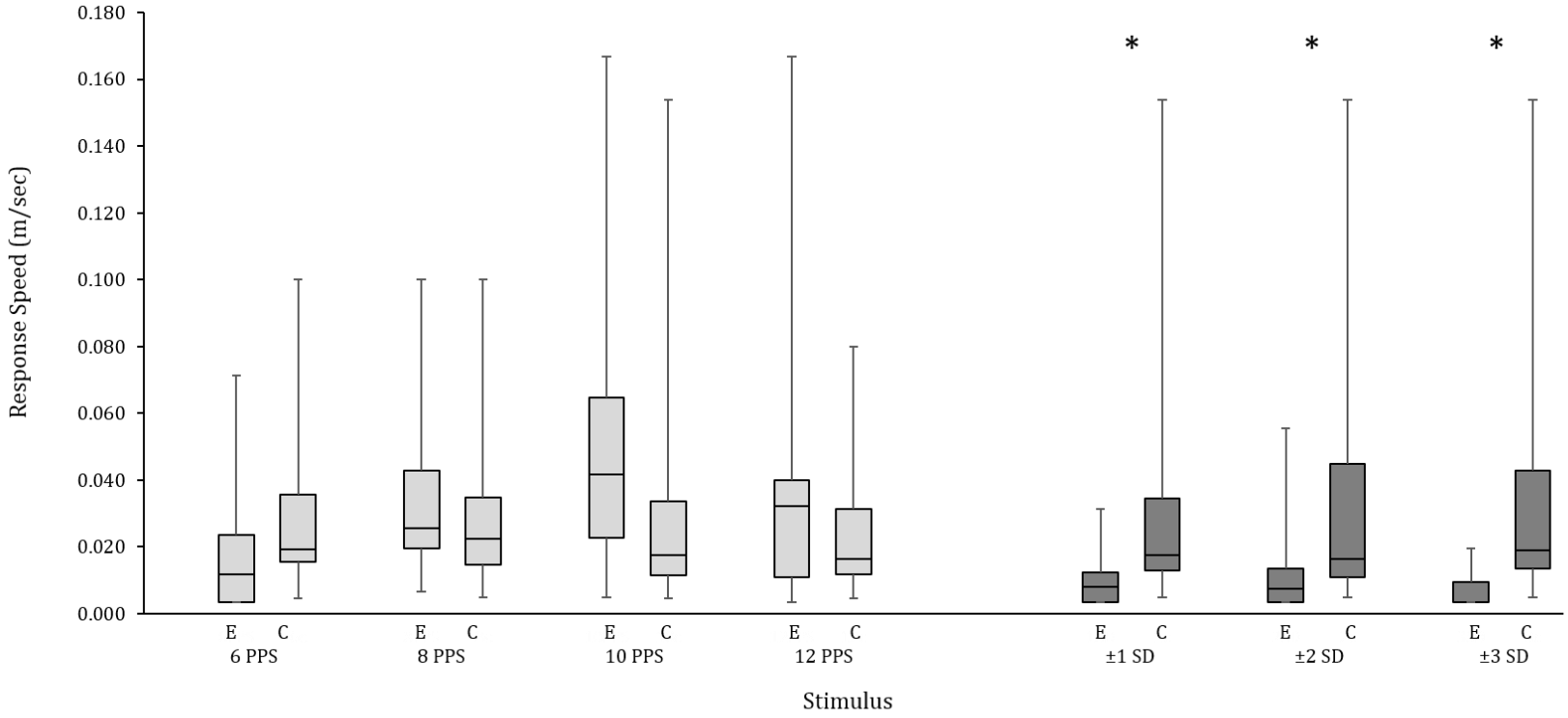

Figure 4-2. Female preference for static and irregular pulse rates

Responses of females to regular and irregular single speaker stimuli. Box plots show the distribution of responses to each experimental stimulus (E) and its corresponding control (C). A (*) indicates experiments in which the distribution of female responses to the experimental test was significantly different than responses to the control.

	Static Pulse Rates				Irregular Stimuli		
	6PPS	8PPS	10PPS	12PPS	1 SD	2SD	3SD
Median response time (sec)	86 (52)	39 (44)	24 (57)	31 (61)	124 (57)	136 (61)	300 (52)
p	0.00782	0.2295	0.1212	0.9165	0.0002*	0.0007*	$3.3X10^{-6}$ *
N	23	23	23	13	23	23	23

Table 4-1. Results of single speaker phonotaxis tests

Responses to the static experimental stimuli were similar to the control; however, females showed significantly slower responses to the irregular experimental stimuli compared to the control (* represents significant differences at Bonferroni corrected alpha = 0.007).

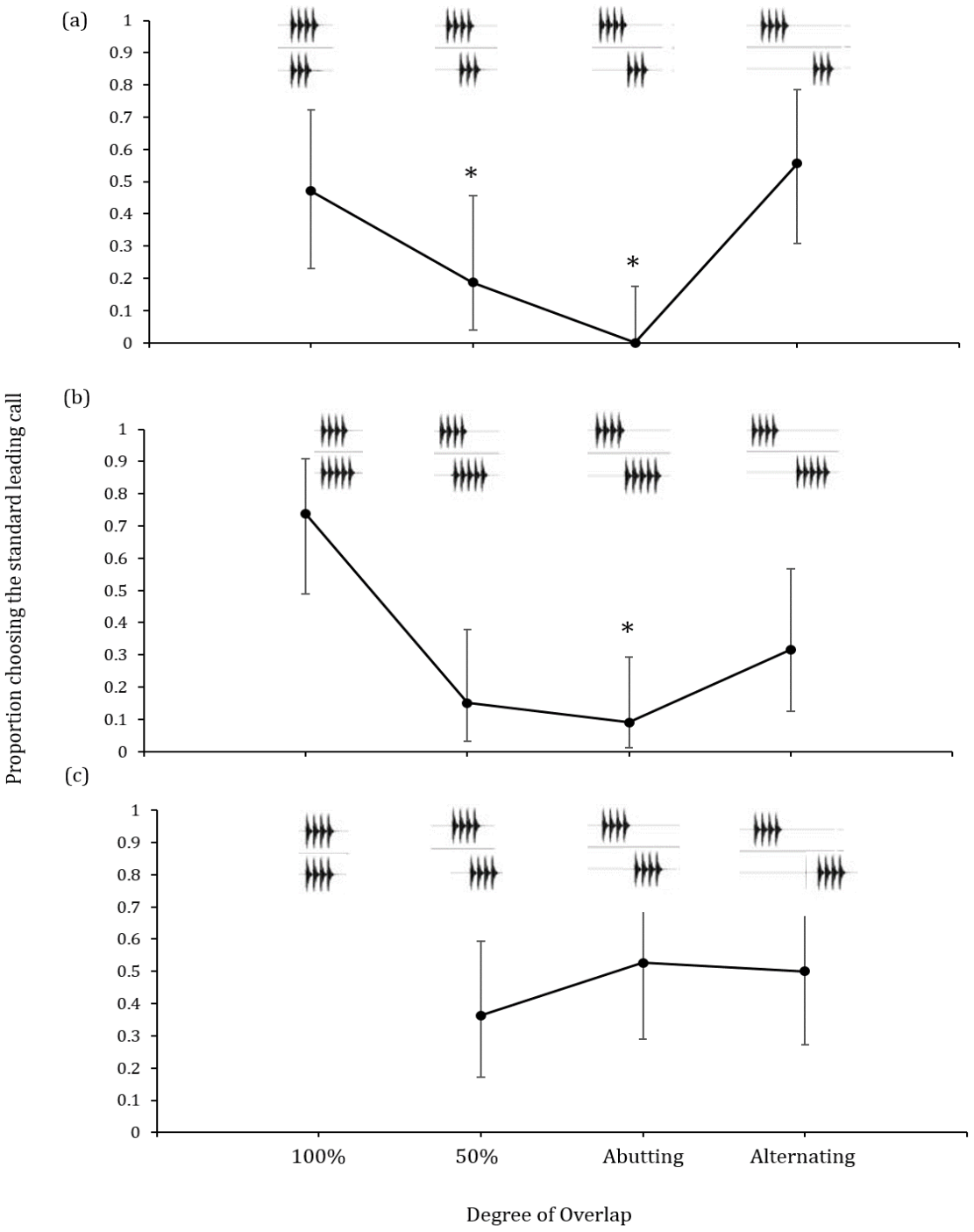
(a)
(b)
(c)
Proportion choosing the standard leading call
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
100%
50%
Abutting
Alternating
Degree of Overlap

Figure 4-3. Female preference for signal timing and sub-pulse number

Preference for signal timing and sub-pulse number. (a) 4 VS 3 sub-pulses, (b) 4 VS 5 sub-pulses, (c) 4 vs 4 sub-pulses (testing signal timing only). Bars are 95% confidence intervals. (*) indicated significance at $p < 0.125$ (Bonferroni correction).

	50 % overlap			100 % overlap		Abutting			Alternating		
	4 vs 3	**4 vs 4**	**4 vs 5**	**4 vs 3**	**4 vs 5**	**4 vs 3**	**4 vs 4**	**4 vs 5**	**4 vs 3**	**4 vs 4**	**4 vs 5**
Proportion Choosing Leader	0.19	0.36	0.15	0.47	0.74	0	0.53	0.09	0.56	0.50	0.32
P	0.021	0.286	0.003*	1	0.064	< 0.0001*	1	0.0001*	0.815	1	0.167
N	16	22	19	17	19	19	19	22	18	20	19

Table 4-2. Analysis of sub-pulse number and signal timing choice tests

Results of forced-choice leader preference and sub-pulse number experiments. (*) indicates significance at Bonferroni corrected alpha = 0.0125.

CHAPTER 5

CONCLUSION

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Chorusing animals provide excellent systems to investigate questions related to the evolution of sexual traits because mate choice and competition are key drivers of selection in these systems (Andersson, 1994; Kirkpatrick and Ryan, 1991). Anurans are especially amendable to such studies because they produce unlearned stereotyped signals which are easy to record and manipulate experimentally. Also, female preference is genetically based and can be quantified using simple, well established experimental designs (Gerhardt and Huber, 2002). Lastly, anurans often gather in complex breeding aggregations where the potential for mismatings is high, and selection for reproductive isolation is expected to be strong (Blair, 1964; Höbel et al., 2003).

The first goal of my dissertation was to investigate the underling genetic basis of the coordination of reproductive behaviors between the sexes. Using the communication system of closely related treefrogs, I used behavioral data to determine if male and female behavioral phenotypes (the acoustic signal and preference for that signal, respectively) were likely to be genetically linked. This is a significant question because understanding the genetic basis of traits related to behavioral isolation can enhance our understanding of population divergence and speciation (Coyne and Orr, 2004). Studies of interspecific hybrids have been integral to our understanding of the mechanisms underlying genetic inheritance. Several hybrid studies lend support to the genetic hypothesis (see Hoy et al., 1977; Doherty and Gerhardt, 1984; Ritchie, 1992; Shaw et al., 2007; Shaw and Lesnick, 2009). Many others have produced contrary findings (see Boake, 1991; von Helversen and von Helversen, 1975; Löfstedt et al., 1989).

In **Chapter 2**, I took an initial step in tracking the inheritance of male signals and female preferences. I crossed two closely related treefrogs, *Hyla chrysoscelis* and *H. avivoca,* and produced a population of F_1 hybrids. Once the individuals reached sexual maturity, we recorded the calls of males and quantified several temporal and spectral features. In addition, we subjected females to preferences tests to determine which signals (the hybrid or the parental calls) were most attractive. All of the males that were recorded produced similar calls. Overall, the signals were somewhat intermediate, but some traits showed obvious trends towards the call of one parent or the other. Interestingly, female hybrids did not prefer the calls of their F_1 siblings. They also showed strong, significant preference in favor of the call of the maternal species and strong discrimination against the paternal call. These results suggest that the hybrid phenotypes are likely due to additive effects of polygenic inheritance with the potential for sex-linkage in some traits. Although we were unable to produce a reciprocal cross, the results provided by our F_1 hybrid align with other studies that suggest that the genes controlling signals and preferences are not tightly linked. In the future, several generations of reciprocal crosses, F_2 and backcrossed generations are needed to determine the role of sex-linked genes in the inheritance of both signal and preference traits. In addition, specific tests to determine the underlying recognition mechanism and strength of preference for each signal feature (pulse rate, pulse duration, call duration, etc.) is necessary (as per Schul and Bush 2002). Ideally, a search for candidate loci underlying these traits is warranted to support the behavioral work initiated here, as seen in the work of Shaw and colleagues (Shaw et al., 2007; Shaw and Lesnick, 2009).

For the rest of my dissertation, I investigated the role of social context (namely male competition) on male sexual signals and female preferences. Signal traits fall on a continuum from highly static to highly variable, and researchers have shown a relationship between the variability found in certain signals and the strength of selection on those traits. Based on behavioral experiments, I investigated both within-male signal variation and female preferences for signal structure and signal timing in order to speculate on the relative importance of signal plasticity and social context on the reproductive behavior in a unique treefrog system.

In **Chapter 3**, I presented the results of studies of male behavior driven by competition. The pine woods treefrog, *H. femoralis* provided an interesting system because males produce a highly plastic, bimodal signal and call from densely populated competitive choruses. My measurements and experiments aimed to understand the extent of signal variation within individuals based on the social context. I found that the variation in the calls of males significantly increased with chorus density. I also recorded isolated pairs of males and conducted several playback experiments with single focal males in order to quantify potential fine-temporal signal adjustments. Males were strongly influenced by the signals of close competitors, and often shifted their own calls relative to the signals of neighbors. The degree of overlap changed often from call to call, and males often settled into bouts of synchrony and alternation. While I found that some males maintained a leading position in more interactions than their partner, in general both males produced signals in a way that allowed at least an unmasked portion of his own call to be exposed. Other studies have shown the prevalence of signal adjustments across a range of signal

modalities (Fischer et al., 2002; Lim and Greenfield, 2007; Zelick and Narins, 1985; Brumm, 2006; Martinez-Rivera and Gerhardt, 2008; Grafe, 1999). Because pulse rate is so variable, it is clear that this aspect of their signaling behavior is not constrained by a rigid central pattern generator, but may be subject to some inhibitory mechanism, resulting in delayed responses to neighboring signalers. Future studies are needed to determine how significant these signal modifications are in terms of reproductive success. I took the first step to address this question by focusing on female preferences in this species in my final research chapter.

In **Chapter 4,** I focused my attention on determining which signal properties were most important for female recognition and preference in *H. femoralis.* My goal was to decipher if females of this species base their mating preferences on species-specific signal features (e.g. sub-pulse structure, irregular pulse periods, etc.), signal timing interactions between individuals, or both. In terms of fine-temporal structure, females responded equally as fast to static pulse rates as they did to the typical bimodal call structure. Finally, when given a choice between leading and following calls, females often choose the stimulus in the following position when the delay was short, regardless of sub-pulse number. These results suggest that masking is likely to be a strong perceptual force driving female preferences in this species. More research is needed to determine the underlying mechanism driving preference, including the specific degree of overlap that elicits this response. This is the first study to investigate signal preferences in this species. Both the male signaling behavior and female preferences represent an interesting departure from the typical behaviors seen in closely related North American hylids.

In complex breeding aggregations such as anuran choruses, individuals compete acoustically and often mask or otherwise degrade each other's signals. The results from my second and third chapters speak to the importance of understanding the role of the social context in order to determine the relative strength of selection on signals and preferences. Despite strong responses to fast, static rates, males of *H. femoralis* continue to produce highly plastic signals within a wide bimodal distribution. It is clear that selection on this particular trait via female choice is weak. Due to the highly complex environment, interactions between individuals may be equally or more important than the species-specific signal features themselves.

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VITA

Jessica A. Merricks was born on November 14, 1985 in Columbia, South Carolina. She graduated from Richland Northeast High School in 2004 and moved to New Orleans, Louisiana to attend Tulane University. She double majored in Environmental Biology and Psychology and conducted research on anole behavior in Bimini, Bahamas as an NSF REU Fellow in her junior year. In 2008, she graduated with a Bachelor of Science degree. In the fall of 2008, she moved to Columbia, Missouri to begin graduate work in the Division of Biological Sciences at the University of Missouri. Under the guidance of Dr. H. Carl Gerhardt, she spent summers at one or more of her field sites in South Carolina, Georgia, Florida, Mississippi, and Louisiana collecting data on treefrog mating behavior. During her time as a graduate student, Jessica enjoyed teaching non-biology majors in an Introductory Biology Laboratory course. She was also granted a National Science Foundation Graduate STEM Fellowship in K-12 Education (GK-12) during which she mentored teachers and taught science at a local elementary school. In 2013, Jessica conducted several science education research projects, one of which received national recognition from the National Association of Biology Teachers. In 2014, Jessica earned her Doctor of Philosophy in Biological Sciences with an emphasis in Ecology and Evolutionary Biology.