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**The Development and Execution of Mate Choice in Túngara Frogs**

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**The Development and Execution of Mate Choice in Túngara Frogs**

**by**

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## **Dedication**

to my family

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I believe that to be a successful behavioral biologist demands a degree of comfort with one's study organism, perhaps even to the point at which one begins to “think” like them—to sense their *Umwelt*. Hence, I owe a great deal to the túngara frogs for their heartiness, harmless disposition, and nightly serenades. For me, becoming familiar with the túngara frog at this level was made possible by four summer field seasons in what became my second home—the dark tropics of Panamá. This in turn was realized through generous funding from the National Science Foundation and the Animal Behavior Society, as well as support from a remarkable staff at the Smithsonian Tropical Research Institute.

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# **The Development and Execution of Mate Choice in Túngara Frogs**

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Interest in the question of when and how species recognition and mate preferences emerge in animals with strong species-typical predispositions has faded since the time of the classical ethologists. In its place, the role of plasticity has surfaced as a central emphasis in the study of animal behavior. Here, I step back and examine the origin and execution of sexual behavior in a tropical frog for which auditory predispositions are key. These experiments challenge assumptions about behavioral development, auditory perception, and stereotyped behavior. First, I illustrate when and how a sex- and species-typical behavior—conspecific phonotaxis—emerges during development. This study demonstrates that phonotaxis, presumably restricted to mature females, is present in both sexes early in postmetamorphic development—potentially long before such behavior might serve an adaptive function. I place this result in the context of hypotheses regarding the development of learned versus non-learned behaviors, and in light of the potential for perception to be altered by physiological changes occurring concomitantly with ontogeny. Next, I describe a set of dynamic mate choice studies that highlight how decision-making in a relatively simple system is more flexible, and less stereotyped, than

was previously assumed. Results here show that frogs temporally update their mate choice decisions in a moment-to-moment fashion as advertisement signals change in real time. By decomposing the decision-making process, I determine the stimulus parameters essential for commitment to an initial phonotactic approach. These studies are followed up by experiments that reveal a high level of individual variation in female choosiness during mate choice. Lastly, I describe a mate choice study that revealed categorical perception in frogs, the first “lower” vertebrate now known to exhibit a perceptual mode previously considered a hallmark of “higher” organisms. Collectively, I make the following arguments: (1) constraints on sensory systems play a larger role in shaping behavior than is generally appreciated, irrespective of the involvement of learning; (2) unstudied sources of variation may contribute significantly to the raw material for sexual selection; and (3) phonotaxis in anurans amphibians is not the simple, stereotyped behavior that has been suggested of it in the past.

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## Chapter 1: Introduction

There are few decisions in life more important than the selection of a mate. Sexually reproducing animals ensure passage of their genes to the next generation by choosing compatible mates, which often rests on species-specific perception of communication signals. The most significant criteria that must be met when selecting a mate is whether the individual belongs to the opposite sex and the same species, as the costs of hybridization are often significant (Dobzhansky 1951, Mayr 1963). Once a female has classified a pool of courting males as belonging to her species, she typically favors some males over others, thus generating sexual selection for attractive traits. Although much research has explored what constitutes “attractive” by determining which particular male traits females use to assess males (Lande 1981, Hamilton and Zuk 1982, Bateson 1983, Andersson 1994), the *process* of reproductive decision-making remains relatively unexplored. Here, I examine this process as it unfolds in the context of communication, using the neotropical túngara frog (*Physalaemus pustulosus*) in a laboratory breeding colony in Austin, Texas and collected from their natural habitat in the Republic of Panamá.

Communication can be defined as information transmission, via a signal, from a sender to a receiver that subsequently influences the behaviors of the animals communicating (Bradbury and Vehrencamp 1998, Gentner and Margoliash 2002). Much of animal communication occurs in reproductive contexts, and for many species the acoustic modality is central to courtship. Males typically produce advertisement signals attracting females that use these signals to localize and select mates. The neuroethological approach to studying acoustic communication has been fruitful, yielding

insight into the organizational features common to the vertebrate auditory system responsible for processing vocalizations in species such as echolocating bats, songbirds, and frogs (Simmons et al. 2002). An early and enduring thrust behind neuroethology's successes was the framework put forward by Tinbergen (1963), which maintained that any behavior could be understood in four essential ways—one can consider the evolutionary history of a behavior, its mechanistic basis, its adaptive significance, and its developmental history within an organism. The latter three foci are explored to varying extents here through the study of auditory behavior in frogs.

#### **DEVELOPMENTAL ORIGINS OF COMMUNICATION**

Developmental studies of communication have largely focused on changes occurring in signalers (production), especially in taxa in which signal acquisition is strongly influenced by learning (Seyfarth and Cheney 1986, Hauser 1989, Tchernichovski et al. 2001, Rose et al. 2004, Hollén and Manser 2007). Fewer studies have examined developmental changes in receivers (perception) (for an exception see Kuhl 1987), and far fewer studies have examined the development of perception for signals that are not learned (for exceptions see Gottlieb 1997). One consequence of such an emphasis is that the discussion of behavioral development has largely become a subset of the biology of learning. The nearly ubiquitous distinction between instinctive and acquired behavior was one that Schneirla, amongst others, warned would dampen the study of developmental biology (Schneirla 1966). Indeed, this focus on learning, as Burghardt (1978, pp 151) aptly criticized when he referred to it as “the only game in town,” deprives us of a broader examination of patterns in development from the perspective of organismal constraints. Such an outlook also carries the risk of considering each type of behavior as depending on a distinct mechanism, a sustained criticism by Schneirla (1946, 1952). Further, given that behavior is considered evolutionarily one of

the most plastic elements of a phenotype, and given that evolutionary change often comes about through the adjustment of developmental programs, it is essential that we examine developmental trajectories of both learned and non-learned behaviors. To this end I examine some assumptions implicit in thinking about certain classes of behaviors as “hard-wired.” In Chapter 2, for example, I present the results of experiments describing the ontogeny of a sexual behavior that motivates new research into the mechanisms underlying the emergence of adult behaviors.

The developmental study in Chapter 2 was motivated by studies approaching this issue from three perspectives. First, studies on songbirds provided evidence that acoustically naïve nestlings (prior to the onset of the sensitive period for song learning) respond more strongly, both behaviorally and physiologically, to conspecific song over alternatives (Dooling and Searcy 1980, Nelson and Marler 1993, Whaling et al. 1997, Braaten and Reynolds 1999, Hauber et al. 2001). With this evidence at hand, Nelson and Marler (1993) suggested that such perceptual predispositions function to minimize heterospecific vocal and auditory learning in oscine passerines. This is important because songbirds learn their vocalizations during a critical period in development and memorizing the song of another species, either for purposes of later production (males) or for mate choice preference as adults (females), would presumably be detrimental to an individual’s reproductive success (reviewed in Marler 1963, Konishi and Nottebohm 1969, Konishi 1985, Catchpole and Slater 1995). This functional explanation of an early perceptual bias implies that such a trait evolved to enable selective attention toward conspecifics; this interpretation would suggest that early perceptual selectivity is restricted to species exhibiting vocal or auditory learning. If this were the case, we would not expect to find such “premature” selectivity in organisms that do not learn their acoustic communication signals. On the other hand, if species that are non-learners do

exhibit ontogenetically early selectivity, this might suggest that the premature expression of perceptual biases is a constraining attribute of developing vertebrate auditory systems. One way to test this prediction would be to examine conspecific predispositions in developing suboscines, for which evidence suggests a lack of vocal learning (Kroodsma and Konishi 1991). Such experiments, however, are complicated by debate about whether suboscines are indeed vocal non-learners (Saranathan et al. 2007), and thus the appropriate selection of a suboscine species would be critically important. Kaspar Hauser experiments have, however, been carried out in túngara frogs and demonstrate that vocalizations and phonotactic responses to them are unaffected by acoustic isolation and stimulation (Dawson 2007, Dawson and Ryan 2009), thus providing an opportunity to test the hypothesis that predispositions to conspecific signals are limited to vocal and auditory learners. To this end, in Chapter 2 I demonstrate the presence of premature selectivity for the advertisement signal in túngara frogs, thus supporting the idea that such predispositions are a more general feature of developing vertebrate auditory systems.

The second line of inquiry motivating the focus of Chapter 2 was a set of physiological experiments that examined peripheral auditory tuning of frogs during postmetamorphic development (Shofner 1981, Shofner 1988, Boatwright-Horowitz and Simmons 1995). Shofner (1981) demonstrated that the developing auditory periphery of bullfrogs (*Rana catesbiana*) becomes more narrowly tuned, and best excitatory frequencies (BEFs) shift to lower values during growth to adulthood—a result supplemented by comparative studies using anuran species of variable body size that show that larger species have enhanced sensitivity to lower frequencies (Narins and Capranica 1976, Shofner 1981, Wilczynski et al. 1984). This finding could be due to developmental changes at several levels, including the following: (1) the external ear (the diameter of the tympanic membrane increases ten-fold during development in bullfrogs;

Shofner and Feng 1981); (2) middle ear transfer functions (smaller frog species have higher upper cutoff frequencies; Saunders and Johnstone 1972, Moffat and Capranica 1978, Pinder and Palmer 1983); and (3) dimensions of the mouth cavity, eustachian tubes, or middle ear cavities (changes in these dimensions affect hearing; Feng 1980, Feng and Shofner 1981, Palmer and Pinder 1984). These types of changes are congruent with the common postnatal course of development in terrestrial vertebrates, wherein following the onset of hearing there are generally decreases in thresholds, increases in the sharpness of tuning, and changes in the most sensitive frequency, until an adult phenotype is reached (Rebillard and Rubel 1981). One potential implication of this result is that the perception of acoustic signals might also vary in concert with these physiological changes in froglets. Despite a call for behavioral studies during postmetamorphic growth in anurans (Shofner 1988), no such studies have been conducted until now. Based on phonotactic responses of juvenile frogs, I show that the perception of conspecific signals is intact and adult-like during postmetamorphic development (Chapter 2). This suggests that despite the likely changes that occur in the peripheral tuning of the auditory system during maturation, perception of species-typical communication signals remains normal. One note of caution—compared to bullfrogs, which undergo increases in body length by up to six-fold (Shofner 1988), túngara frogs exhibit less relative change in body size during postmetamorphic ontogeny (approximately four-fold change in body length). It is possible, therefore, that changes in peripheral tuning are more exaggerated in bullfrogs and therefore do affect perception. Such a study would be a worthwhile future pursuit.

Lastly, the pioneering work of G. Gottlieb in evolutionary-developmental biology (“evo-devo”) inspired aspects of my dissertation research by framing this issue of learned versus acquired traits in a new light. Gottlieb’s studies of perceptual abilities in ducklings

suggested that conspecific preferences for the maternal assembly vocalization rest, oddly enough, on experience hearing (self) embryonic contact vocalizations before hatching (for review see Gottlieb 1997). This result suggested that despite a lack of vocal learning in the various bird species that Gottlieb and his colleagues studied, the normal species-typical responses relied heavily on experience—in this instance, self-stimulated experience. As such, the traditional distinction between learned and acquired characteristics began to break down conceptually. One of the many outcomes of this research was the recognition that “environment” (i.e., “nurture”), as it is traditionally construed, cannot be extracted from genes during ontogeny. This notion was perhaps best stated by Hebb (1953) when he argued that it is as meaningless to ask how much a given piece of behavior rests on genes and how much on the environment as it is to ask how much the area of a field depends on its length and how much depends on its width. As a relatively new field, evo-devo attempts to understand the evolutionary and developmental origins of phenotypes, and in doing so has borrowed a variety of techniques and concepts from, among other fields, comparative genetics, cell and molecular biology, physiology and morphology. What has been largely absent from evo-devo is an emphasis on behavior (for exceptions, including the original conception of developmental canalization by E. B. Holt, see Holt 1931, Gottlieb 1997, Gottlieb 2003, Robert 2003).

As with many aspects in biology, much can be said about the utility of reconsidering discrete classifications as belonging instead to a continuum. One criticism of the evo-devo philosophy is that it is often vague, focusing on psychological constructs rather than empirical work. At one level, the nature-nurture debate comes down to differences of scale—no one believes that genes in a vacuum (i.e., in the absence of the endogenous and exogenous signals or activity) can sufficiently explain phenotypes. However, this does not preclude a conceptual framework that identifies which features of

the environment (social, physical, internal) constrain or liberate characteristics of the developing phenotype. One of the advantages of examining the developmental origins of species recognition in the túngara frog is that, in addition to exhibiting neither vocal nor auditory learning, these animals lack vocal behavior prior to adulthood (Dawson 2007). Therefore, unlike the ducklings studied by Gottlieb, we can be certain that vocalizations, including from self-stimulated sources, play no role during postmetamorphic development. Thus, we can perhaps view ontogenetically early species recognition in túngara frogs (Chapter 2) as representing a point on the continuum of experience-dependent behavior; for conspecific auditory behavior, túngara frogs are situated at the experience-independent end of the spectrum.

## **BACKGROUND**

### **Anuran Acoustic Communication and Sexual Behavior**

The vertebrate class Amphibia consists of three orders: the Urodela (salamanders and newts), the Gymnophiona (caecilians), and the Anura (frogs and toads). Anurans are by and large the most vocal of the amphibians, hence the extensive attention paid to them by biologists interested in the mechanistic and evolutionary basis of vocal communication (reviewed in Ryan 2001, Narins et al. 2007, Wells 2007). As such, anurans offer a unique opportunity to shed light on the mechanisms that make auditory recognition possible, and approaching this issue developmentally provides a window into the formation of intrinsic selectivity. In nature, adult male frogs produce advertisement calls during a temporary breeding season, attracting gravid females to approach (i.e., exhibit phonotaxis) and mate. Phonotaxis is an unlearned behavior—no animal training is required, and the stereotypic nature of the calls and consistency of phonotactic preferences has led to the suggestion that experience plays virtually no role (Gerhardt and

Huber 2002). In Chapter 2, I examine the development of conspecific phonotaxis in the túngara frog, *Physalaemus pustulosus*—the only anuran species in which the absence of vocal and auditory learning has been confirmed directly through acoustic isolation experiments, demonstrating that neither vocalizations (males) nor phonotactic responses to them (females) are affected by conspecific acoustic isolation or by heterospecific, conspecific, or noise stimulation (Dawson 2007, Dawson and Ryan 2009). Thus unlike studies of songbirds, frogs do not have to be socially and acoustically isolated to examine the genetic contribution to behavior. Likewise, the anuran brain is relatively simple, but at the same time anurans and mammals share related structures. Together the simplicity of the acoustic signals, robustness of the behavioral responses, and tractability of the nervous system that decodes these signals makes the anurans ideal for studying the development of auditory recognition.

Much of what is known about anuran behavior comes from studies of mate advertisement behavior and female choice in adults at breeding aggregations and in the laboratory (reviewed in Ryan 2001, Wells 2007). A large literature is also devoted to the investigation of larval behavior and physiology (reviewed in McDiarmid 2000). Despite this interest in larval and adult forms, only a small number of studies have examined the physiology of audition in subadult frogs, and even in these instances only basic audiograms using simple stimuli have been used to describe gross sensitivity of the peripheral auditory system (Hetherington 1992), eighth nerve (Shofner and Feng 1981), and torus semicircularis (Boatwright-Horowitz and Simmons 1995). I am unaware of any published studies on auditory responses to communication signals or behavioral responses to sound in subadult frogs. This paucity of research on subadult anurans is in part due to the difficulty of studying young animals in the field, but also the result of a relative lack of interest in the behavior of this class of organisms when they are young.

Consequently, there are a variety of untested assumptions regarding young anurans, such as the notion that they are simply “miniature adults.” This position, for example, is in conflict with another assumption regarding behavior, including sexual behavior such as phonotaxis—that such behavior will be exhibited only when there is an adaptive function for the behavior (e.g., in sexually mature adults). On the one hand, if subadult anurans are simply small versions of adults, then processing and responding in a species-typical manner to acoustic communication signals might appear, though dampened, in subadults. Such an outcome would, however, conflict with the notion that an archetypical sexual behavior such as anuran phonotaxis is limited narrowly to developmental stages for which such behavior functions in an adaptive manner (to choose a mate). The idea that responses will only appear developmentally when they are adaptive potentially underestimates the role of constraints in shaping neural and behavioral responses to social and environmental stimuli. By constraints, I mean any element of the phenotype or genotype that biases or limits the breadth of developmental outcomes achievable. In Chapter 2 I address, and partially resolve, this conflict by examining developmental trajectories of anuran phonotaxis.

### **The Túngara Frog**

The túngara frog is a small, abundant leptodactylid (ca. 30 mm snout-vent length, SVL) distributed throughout much of Central America (Weigt et al. 2005) that has been the subject of a large volume of research (reviewed in Ryan 1985, Ryan and Rand 2003, Ryan in press). Much of what is known about this species comes from studies of sexual behavior in mature adults collected from, or studied in, the field. Adult males and females unite for a relatively short window of time (hours) to mate at breeding aggregations (temporary pools of water). Males externally fertilize a female’s eggs while producing a foam nest that will rest on the water surface (Heyer and Rand 1977). Female túngara

frogs exhibit a synchronous-asynchronous pattern of oogenesis, which has been suggested to function as a mechanism to maximize fecundity under variable environmental (e.g., pond availability) conditions (Davidson and Hough 1969). A few days after oviposition, the fertilized eggs hatch into tadpoles, which live aquatically for the first few weeks before metamorphosing into terrestrial froglets. At the completion of metamorphosis froglets have approximately 25% the snout-vent length (SVL) and 5% the body mass of mature adults (see Chapter 2). Postmetamorphic growth to adulthood commences at this time and continues for approximately nine months at which point adults can mate.

During the breeding season (principally May through December), males vocally advertise to females using a species-typical call, known as the “whine” or simple call (Ryan 1985). This call consists of a harmonically related stack of frequency sweeps with the dominant frequency (which is also the fundamental for most of the call) beginning at approximately 1000 Hz and terminating at about 400 Hz and is about 300 milliseconds in duration. Males can produce a complex advertisement call by ornamenting the whine with one to seven suffixes known as “chucks,” producing what are known as “whine-chuck” calls (Ryan 1985, Bernal et al. 2007). The chuck is also harmonically structured but unlike the whine the higher frequency harmonics contain more energy than the lower frequencies with most of the energy above 1500 Hz. A single chuck is much shorter than the whine, typically 30 milliseconds in duration. Although male call repetition rates vary, especially at the beginning of a bout, most males achieve a relatively stable call rate of 1 call every 2 seconds (Ryan 1985). The peak sound pressure level (SPL) of the male call is loud, registering approximately 90 dB SPL at 50 centimeters (Ryan 1985).

Females use advertisement calls to localize and select a male amongst a chorus. By making physical contact with a male, females select a mate, after which the male

mounts and clasps the female in a posture known as amplexus (Ryan 1985). Although the whine is necessary and sufficient to attract adult females to mate, the complex call is preferred. Using reproductively mature females collected in amplexus in the field and tested in laboratory two-choice phonotaxis tests, a large set of studies have confirmed that the whine-chuck calls are strongly preferred to whine calls with preference strength of approximately 85% (see Ryan and Rand 2003a). While these reproductively mature females respond with robust phonotaxis towards conspecific mating calls, adult females in-between breeding cycles (i.e., post-mated) exhibit a decrement in phonotactic responsiveness yet retain recognition and discrimination abilities (preference for whine-chuck over whine; Lynch 2005). By injecting these post-mated females with gonadotropins, robust phonotactic behavior can be rescued (Lynch 2005).

Additionally, when females are presented with a synthetic whine that is acoustically intermediate between the conspecific and a heterospecific whine, they occasionally err, falsely recognizing this signal as conspecific (Ryan et al. 2003). This type of testing condition enables one to examine female permissiveness using stimuli of subtle variation. Female túngara frogs also exhibit strong preferences for calls of higher amplitude over lower amplitude alternatives, presumably resulting in attraction towards nearer males and thus reducing the travel time and distance required to reach the chosen male (Ryan and Rand 1990). As described in Chapters 3 and 4, I used these preferences for complex over simple and high- over low-amplitude to examine, in detail, the timing of decision-making as females are in the process of mate selection.

While females prefer complex to simple calls, for a given complex call (natural or synthetic) they do not appear to prefer a more complex version at the same amplitude (e.g., whine with 4 chucks) to a less complex version (e.g., whine with 2 chucks) (Ryan

unpublished data). In Chapter 3 I demonstrate that it is possible that such a preference is present but that it is exhibited under different testing conditions.

Phonotaxis in male anurans is examined less frequently, and it is assumed that in chorus breeding species (e.g., túngara frogs) this behavior functions to guide males to aggregations for the selection of a calling site and in territorial species to repel rival male intruders (Hödl et al. 2004). Under standard (i.e., static) playback conditions, male túngara frogs exhibit phonotaxis towards the conspecific advertisement signals and, as with females, prefer the complex call with similar preference strengths (Chapter 2, Bernal et al. in press (a)). In Chapter 3 I present results from a dynamic phonotaxis experiment that used males to determine if they exhibited updating behavior similar to that seen in females. In females, selection of the complex call is an expression of mate preferences—in males, such behavior might increase the likelihood of selecting a high quality calling site, because complex calls are indicative of high density choruses which confer increased per capita mating success for males and lower predation risk (Ryan et al. 1981, Bernal et al. 2007).

Individual male túngara frogs differ in the attractiveness of their calls. Ryan and Rand (2003b) studied female mate choice in response to recorded natural calls from males in the study population (Gamboa, Panamá) and showed that the complex calls of some males are consistently more attractive than those of others. I used this natural variation in attractiveness to examine how such intrinsic differences influence the extent to which females commit to a given male in the face of dynamic changes in call complexity (Chapter 3).

### **The Frog Auditory System**

The frog auditory system has played a key role in hearing research, advancing our understanding of properties such as population coding, complex feature detectors, and

plasticity (reviewed in Fritzsche et al. 1988, Goense and Feng 2005). Unlike the mammalian inner ear with its single basilar membrane, frogs have two auditory end organs, the amphibian papilla (AP) and the basilar papilla (BP), which are physically separated and tuned to different frequency ranges—the AP is tuned to low and mid-frequencies and the BP to high frequencies (Zakon and Wilczynski 1988). By virtue of this tonotopy, the inner ear of the túngara frog is differentially stimulated by the two call components, with the whine stimulating principally the AP and the chuck stimulating principally the BP.

Eighth nerve afferents terminate in the dorsolateral nucleus (DLN) of the medulla. A superior olivary nucleus (SON) receives ascending inputs from the DLN and a lateral lemniscus connects these medullary nuclei with the auditory midbrain, torus semicircularis (TS). The TS is a large structure homologous with the mammalian inferior colliculus and has been subdivided into the following four nuclei: principal, laminar, ventral, and midline (Hoke et al. 2004). The principal nucleus is the primary recipient of ascending auditory input while the laminar and ventral nuclei receive some ascending afferents from caudal brainstem auditory nuclei but extensive descending inputs from thalamic nuclei. In addition to the afferent connections, the efferent projections from these toral subdivisions differ. The principal nucleus gives restricted ascending and descending projections, while the laminar and ventral nuclei give robust descending and ascending projections, thus serving as the main output stages for the TS (Feng and Lin 1991). The role of cells in the midline nucleus is unclear but has been shown to differentially respond with IEG activity following sound stimulation (Hoke et al. 2004).

The response properties of TS neurons are strikingly complex and diverse compared to neurons in the mammalian inferior colliculus, and it is likely that a good deal of auditory processing has been accomplished at the level of the TS. In fact, even

small lesions of the TS in female gray treefrogs (*Hyla versicolor*) nearly abolish phonotactic responses, while significant lesions of the auditory thalamus have little effect on phonotaxis, demonstrating the importance of the TS not only in processing sounds but also in mediating acoustically-guided behavior (Endepols et al. 2003). Indeed, this might be expected of an auditory system devoted to processing only a few behaviorally relevant communication signals (Fuzessery 1988).

It has been suggested that steroid hormones might contribute to auditory sensitivity. In female plainfin midshipman fish (*Porichthys notatus*), auditory sensitivity to and temporal coding of the male advertisement vocalization is enhanced during the breeding season, and this auditory coupling can be induced outside the breeding season by the application of either testosterone or 17 $\beta$ -estradiol (Sisneros 2004). Support for this idea has been found also in anurans—steroid receptors have been found in the TS (laminar nucleus) of the aquatic frog, *Xenopus laevis* (Kelly 1982), and recently estradiol receptors have been identified in the TS of adult female túngara frogs (S. Burmeister pers comm), and injection of estradiol has been shown to elevate phonotaxis in post-mated female túngara frogs (Chakraborty and Burmeister 2009). Further, intraventricular injections of 17 $\beta$ -estradiol to the TS of adult female northern leopard frogs (*Rana pipiens*) results in increases in the amplitude of auditory evoked responses, suggesting that the TS becomes more sensitive to sounds in the presence of estradiol (Yovanof and Feng 1983). These results indicate a role for gonadal steroid hormones in modulating auditory system function.

Many features of the anuran auditory system are adult-like by the time metamorphosis is complete, including cholinergic- and GABA-immunoreactivity patterns and androgen receptor distributions (Gorlick and Kelley 1986, Kumaresan et al. 1998, Simmons and Chapman 2002). In other instances, however, the physiology of the

auditory system undergoes postmetamorphic changes, including changes in the tuning of eighth nerve afferents and toral sensitivity (Shofner 1988, Boatright-Horowitz and Simmons 1995). Neither behavioral nor neural responses to social signals during postmetamorphic development in anurans have been examined previously.

## **RELATIONSHIPS AMONG CHAPTERS**

The research presented in this dissertation focuses largely on unexplored elements of a popularly studied behavior—phonotaxis in frogs. I have centered my attention largely, though not exclusively, on the temporal dimension of this particular stimulus-elicited behavior. By examining when phonotaxis emerges during development, and exactly when and how such responses are executed in adults, I simultaneously address a suite of questions relevant to behavioral biology, including recurrent themes such as the role of developmental constraints, individual differences, and the need to closely re-examine what are all too often typological ways of thinking about animal behavior. Chapter 2 follows on the heels of this chapter by describing a series of ontogenetic studies that detail the emergence and subsequent rise of acoustically-guided behavior, beginning with an immediate-early gene study in túngara frogs that suggests there are both behavioral and neural predispositions to the species-typical advertisement call in subadult animals. Chapter 2 also describes a variety of sex differences, including the emergence of close-range perseverance behavior in females as they near an approaching male. This is an example of short-distance communication behavior—an increasingly important area of study in animal communication (see Cator et al. 2009).

In Chapter 3, the lengthy ontogenetic timeline is replaced by the moment-to-moment timelines observed in reproductive adults immediately engaged in mate selection. Here, I treat decision-making as a dynamic process, and by manipulating the acoustic stimuli presented to adults in real time I isolate the stimulus parameters essential

for commitment to a mate choice. Chapter 4 pursues these findings further by probing the extent of individual differences in temporal updating behavior, thus illustrating the range of choosiness present in the female population and relating such variation to body condition differences and possible adaptive explanations.

Lastly, in Chapter 5, I present findings from experiments aimed at determining the precise perceptual mode that underlies conspecific signal recognition in adult females. Categorical perception occurs when continuous and variable stimuli are perceived as belonging to discrete categories whose elements resemble each other more than they resemble elements belonging to other categories. The vast majority of studies of categorical perception have focused on humans, and the perception of speech sounds in particular (reviewed in Harnad 1987). A popular theory to explain categorical perception, the motor theory of speech perception (Liberman et al. 1957), has been criticized for a variety of reasons, chiefly because it limits categorical perception to humans and specifically to the auditory domain. The motor theory suggests that discontinuities in speech perception are due to the parallel discontinuities in speech production. An example of this would be that the auditory discontinuities between stop-consonant categories, such as /ba/ and /da/ occur because of the discontinuities required to pronounce them. If categorical perception is present, however, for non-speech sounds (including in non-human animals), then the explanatory power of motor theory is acutely limited. Another criticism of categorical perception studies, particularly amongst ethologists (see Ehret 1987), is that the stimuli employed typically vary continuously in only one dimension, yet natural stimuli tend to vary in multiple dimensions. Result from Chapter 5 support the idea that frogs, like several other animal species including humans, are capable of perceiving continuous variation in signals categorically. This study provides the first demonstration of categorical discrimination in a “lower” vertebrate and

in a female mate choice context (for an example of categorical perception of mating signals by male swamp sparrows see Prather et al. 2009). This result is especially relevant as the signals used straddle the boundary between conspecific and heterospecific calls, and therefore has important implication for mechanisms of species recognition. If categorical perception functions to exaggerate the differences between naturally meaningful categories (and collapse differences within categories), then one such domain to search for examples in non-human animals would be species recognition contexts wherein the cost of recognition errors can be quite high. Moreover, I use stimuli that vary multidimensionally and examine the natural acoustic space of male calls in the study population, making the external validity of the results that much more pertinent.

As a whole, the studies presented here force a reappraisal of our expectations about behavioral complexity. If we accept a popular definition of stereotypic behavior—that it is a relatively invariant mode of behavior elicited or determined by a particular stimulus—then the claim that “the social behavior that acoustic signals elicits [sic] in frogs also consists of a small stereotyped repertoire...” (from Wilczynski and Ryan 1988) seems an outmoded position. Instead, the studies outlined here illustrate the variety and complexity of anuran phonotactic behavior, and the utility of this system for assessing the development and execution of such decision-making behavior.

I wrote Chapters 2–5 to be published as individual papers in scientific journals (Chapter 2: see Baugh and Ryan in review (a); Chapter 3: see Baugh and Ryan in review (b); Chapter 4: see Baugh and Ryan in review (c); Chapter 5: see Baugh et al. 2008), and as such there are some redundancies between chapters, particularly in background material and the description of methods. I have included a relatively large set of figures to accompany the results because as a critic I appreciate visual depictions of data and because different readers may be interested in results that speak to diverse questions.

## **Chapter 2: The Development of Sexual Behavior in Túngara Frogs**

### **ABSTRACT**

The question of when and how species-typical behavior arises during development has important implications for the evolution of communication systems. In anurans, acoustic communication is a central component of sexual behavior and therefore has been studied in mature adults. This study examined the emergence of a critical component of sex, response to sexual signals—phonotaxis—in male and female frogs. An experiment using the immediate early gene response and behavior suggested a developmentally early expression of species recognition. I followed this study with a within-subject behavioral study aimed at determining the ontogenetic trajectories of species- and sex-typical phonotactic responses as animals developed from metamorphic froglets to reproductive adults. The results demonstrated that species-typical phonotaxis emerges quite early during postmetamorphic development, well before sexual maturity. Phonotactic responses of adults indicate an overlapping yet sexually distinct set of behaviors. The results suggest that a developmentally early bias in the auditory system for species-typical signals might be a more general phenomenon than previously thought, and that the neural circuits responsible for processing and responding to conspecific advertisement signals in a species-typical manner might develop long before the coordinated behavior is demanded of the organism.

### **INTRODUCTION**

An inclusive investigation of behavior must consider at least four major features—evolution, adaptation, causation, and development (Tinbergen 1951, Tinbergen 1963, Bolhuis and Verhulst 2009). One issue in animal communication that unifies

Tinbergen's approaches is vocal recognition (Gentner and Margoliash 2002, Ryan 2005). For a signal to transmit information effectively the receiver must be able to interpret predictably some of the variability in the signal. An intrinsic bias in sensory organs and the central nervous system for detecting and perceiving conspecific vocalizations can aid in this process, contributing to physiological parity between sender and receiver. In this way, animals often exhibit behaviors with strong predispositions to specific sensory stimuli that are likely to result from the activation of developmentally programmed circuits (Gottlieb 1965, Gottlieb 1991, Balaban 1997, Long et al. 2001). While past arguments about the nature-nurture dichotomy have yielded to a more inclusive consensus that organisms represent a complex union of plastic and fixed traits, we lack examples in animal communication of behavioral development in receivers that occurs in the absence of learning. Examining when a coordinated behavior first emerges, rather than when it is first exhibited in its full form, is especially relevant for behaviors that result from stimulus processing, and in particular stimuli of social relevance such as species specific mating signals, because it also informs our understanding of the emergence of species recognition (Burghardt 1977).

Many animals exhibit certain behavioral predispositions, and these are often expressed during different developmental stages. Vocal recognition in songbirds is one example in which auditory predispositions are thought to prepare animals for communication later in life. Shortly after the seminal discovery by Thorpe (1958) and Marler (1963) that songbirds acquire their vocalizations through vocal learning, it became apparent that, in addition to an experience dependent aspect of vocal behavior, there were also important predispositions (Thorpe 1961). Most of these early investigations concentrated on the sender by examining the song produced by adult males as a function of early experience (Marler 1998). There were, however, some studies of early

development of song recognition. In these studies, physiological (e.g., cardiac orienting response) and behavioral (e.g., begging call rate) measures demonstrated that naïve fledgling songbirds respond more strongly to conspecific song compared to alternatives such as heterospecific song (Dooling and Searcy 1980, Nelson and Marler 1993, Whaling et al. 1997, Braaten and Reynolds 1999, Hauber et al. 2001). In white crowned sparrows, this bias for conspecific song appears to be based on selectivity for acoustic features (e.g., frequency) rather than more complex features such as syntax (Whaling et al. 1997). In songbirds it has been proposed that such biases function to guide conspecific vocal learning (Nelson and Marler 1993). This functional suggestion, however, would likely not explain biases in animals that lack vocal and auditory learning. Similarly, psychological studies have shown that human neonates respond preferentially to human vocal sounds (Eimas et al. 1987), and even express unlearned preferences for consonant over dissonant musical sounds (Zentner and Kagan 1996).

Although there is a good understanding of auditory ontogeny in the context of learned responses (e.g., birds, humans), there is little known about how the expression of responses to acoustic signals proceeds when there is a lack of learning. This is a major oversight as there is little evidence that learning is involved in shaping acoustic pattern recognition in many major taxa that utilize acoustic communication, such as insects (Greenfield 2002; for an exception see Bailey and Zuk 2008), frogs (Gerhardt and Huber 2002), most non-passerine birds (Catchpole and Slater 1995) and most primates (Newman and Symmes 1982; for exceptions see Seyfarth and Cheney 1999). Thus in these taxa we do not even know if predispositions to conspecific sexual signals are expressed behaviorally in the absence of vocal or auditory learning, or if any sex differences seen in adults are manifest ontogenetically.

In the present study, phonotaxis in frogs was used as a behavioral measure of acoustic pattern recognition analogous to the approaches used in avian and human recognition studies. Phonotaxis in reproductive female anurans has been used extensively to assess mate choice preferences, species recognition and signal detection (Ryan 2001, Gerhardt and Huber 2002). Phonotaxis in male anurans is less frequently examined and it is assumed that in chorus breeding species this behavior functions to guide males to aggregations and in territorial species to repel rival male intruders (Hödl et al. 2004). While there are exceptions (Bush et al. 1996, Shen et al. 2008), it is uncommon in anurans for males to approach vocalizing females. Sex differences in phonotactic responsiveness and underlying locomotor differences have not been examined previously. Importantly, phonotaxis is an unlearned behavior; no animal training is required and the stereotypic nature of the calls and consistency of phonotactic preferences have led to the suggestion that experience plays virtually no role (Gerhardt and Huber 2002). In the present study, I examined the development of conspecific phonotaxis in the túngara frog, *Physalaemus pustulosus*—the only anuran species in which the absence of vocal and auditory learning has been confirmed directly through acoustic isolation experiments, demonstrating that neither vocalizations (males) nor phonotactic responses to them (females) are affected by conspecific acoustic isolation, heterospecific, conspecific, or noise stimulation (Dawson 2007, Dawson and Ryan 2009). Thus unlike studies of songbirds, frogs do not have to be socially and acoustically isolated to examine the genetic contribution to behavior. Therefore, responses to sexual signals by sexually immature individuals, or premature responses, might suggest that intrinsic biases are a more general feature of the vertebrate auditory system than has previously been appreciated.

Despite the prevalence of auditory biases, there are significant lacunae in our understanding of their developmental bases, including sex differences in the expression of such predispositions (Dooling and Searcy 1980, Nelson and Marler 1993). Given the extent of research on túngara frog adults (reviewed in Ryan 1985, Ryan and Rand 2003a, Ryan in press), we are positioned to address questions regarding the development of auditory, and in this case, sexual behavior. As a system, túngara frogs provide an important advantage—a stimulus-elicited behavior for a task relevant to sexual reproduction is shared by both sexes, making possible the dual examination of behavioral ontogeny in both males and females. This is the first study, to my knowledge, to examine phonotaxis in subadult anurans. I propose two competing hypotheses for the developmental time course of phonotaxis and I refer to these as “graded” and “threshold” patterns. First, if the auditory and motor circuits for processing and responding to species-typical signals are present after metamorphosis and phonotactic responses to conspecific signals are simply a function of circulating gonadal steroid levels, then phonotactic responses might emerge early in development when the gonads begin secreting hormone, and increase in frequency gradually as animals grow to adulthood. Alternatively, phonotaxis might only be expressed at sexual maturity, either because the circuitry for this behavior is absent in sexually immature animals or because only at sexually maturity does a threshold level of steroid content activate this circuitry and motivate responses.

### **The System**

During the breeding season (May–December) adult male túngara frogs vocally advertise to females using a species-typical call, known as the “whine”, or simple call (Ryan 1985). Males can embellish facultatively the whine with one to seven suffixes known as “chucks” thereby producing what is known as the complex call. These whine-

chuck calls are preferred compared to whines in two-choice phonotaxis tests by reproductively active females collected in the field (Ryan 1985, Ryan and Rand 2003a). While these reproductively mature females respond with robust phonotaxis towards conspecific mating calls, adult females in-between cycles of ovulation (“nonreproductive”) also exhibit recognition and discrimination of conspecific signals, although the overall frequency of phonotactic responses is diminished (Lynch et al. 2005a). Further, reproductive adult males collected in the field have been shown to exhibit positive phonotaxis to conspecific mating calls with a similar preference for the complex call (Bernal et al. in press (a)).

Examining juvenile behavior prior to reproductive maturity will provide a window into the origin of auditory recognition. Here, I pursue early recognition by tracking acoustically guided behavior during postmetamorphic growth in male and female frogs to determine the developmental time course of this sexual behavior, which is critical to species recognition, and furthermore to examine sex differences therein. I began by characterizing the fully developed behavior more closely in adults so that there is a standard with which the behavior of the developing animals can be compared (Bekoff 1978).

### **Immediate Early Genes and Acoustic Communication**

Immediate early genes (IEGs) have been used successfully as markers of neuronal activity in a variety of species and systems, and represent the initial genomic response to an inducing stimulus. Much of IEG research in the nervous system has focused on a small handful of genes—one of the most familiar of which is *egr-1* (Mello et al. 1992). Action potentials and *egr-1* expression are often linked because both can be induced by the same neurotransmitters (Clayton 2000). Induction of *egr-1* mRNA or protein in the auditory system has been an effective measure of neural activation in response to acoustic stimuli

in songbirds (Mello et al. 1992, Clayton 2000). Peak *egr-1* expression is typically achieved approximately 30 min after sound stimulation (Clayton 2000). For this reason it is possible to infer a connection between increases in *egr-1* expression and recent increases in synaptic transmission. In this way *egr-1* expression has been used to map functional activity of brain regions (Jarvis 2004). This technique was applied to adult male túngara frogs to map patterns of activation in the TS in response to various natural sounds, and the results showed stimulus-specific patterns of *egr-1* induction across subdivisions of the TS (Hoke et al. 2004). Furthermore, when considering activation across TS subdivisions, a discriminant function analysis demonstrated significant differences in *egr-1* levels in response to the various acoustic stimuli and correctly classified approximately 70% of animals to the sound stimulus they heard (Hoke et al. 2004). This result demonstrates that there is enough potential variation in midbrain activation to discriminate between these natural sounds in adult males. In another study it was shown that there is an increase in the magnitude of *egr-1* mRNA induction in the laminar nucleus of the TS of non-reproductive adult female túngara frogs that have received systemic injections of human chorionic gonadotropin (hCG) hormone and stimulation with the conspecific call (Lynch and Wilczynski 2008). Together, the results of the *egr-1* studies in túngara frogs suggest that hearing the advertisement call leads to a distributed IEG induction across the subdivisions of the TS and this pattern is sensitive to the reproductive state of the animal. Because hCG might have many actions, including the stimulation of steroid hormone production in the gonads as well as direct effects in the brain as a ligand for leutenizing hormone receptors, the mechanism of action responsible for inducing behavioral and neural responses to the advertisement call in female túngara frogs is unknown. It is conceivable, however, that gonadal steroid hormones which are elevated in several conditions, including during the breeding season,

following hCG injection, as well as simply following repeated stimulation with the advertisement call, are chiefly responsible for this induction (Lynch and Wilczynski 2005, Lynch et al. 2005a, Lynch et al. 2005b, Lynch and Wilczynski 2006). Likewise, steroid hormones might help explain developmental changes in female phonotaxis behavior and brain activation patterns.

## **METHODS**

### **Experiment 1: Behavioral and Immediate Early Gene Responses to Sound in Juvenile Túngara Frogs**

Animals were raised from a captive breeding colony housed at the University of Texas at Austin and were age-matched at approximately four months post-hatching. The sex of frogs in this experiment was unknown. Animals were randomly assigned to one of the four following acoustic treatments: (1) *P. pustulosus* whine, (2) *P. petersi* whine, (3) white noise, and (4) silence (Figure 1a, f, e). A 2-hour period of silence and dark adaptation preceded each acoustic presentation. This was intended to reduce differences in endogenous auditory activity between animals and allow for baseline levels of *egr-1* mRNA to be reached.

#### ***Stimuli***

The *P. pustulosus* whine used here is a descending frequency sweep of ca. 300 ms in duration with its dominant frequency component ranging between ca. 1000 Hz and 500 Hz. This whine was recorded from a calling male at a field site in Panamá and has the average acoustic features for calls of the study population and has been used in other studies (Figure 1a; see Ryan and Rand 2003b). *P. petersi* is an allopatric congener in the *Physalaemus pustulosus* species group that occurs west of the Andes in Ecuador and Peru; the whine from this species was recorded from a calling male in Ecuador and

consists of a descending frequency sweep of ca. 150 ms in duration with a dominant frequency ranging between ca. 1000 Hz and 350 Hz (Figure 1f). The noise stimulus was identical to the noise stimulus used in Experiment 2 of this study (Figure 1e; see Experiment 2 for description of synthesis). All sound stimuli were matched for the peak amplitude of the *P. pustulosus* whine. Stimulus files were broadcast (CoolEdit, Syntrillium Software, Scottsdale, AZ, USA; sampling rate of 44.1 kHz and 16 bit depth) at a rate of 1 call per 2 seconds through speakers at 82 dB SPL at 45 cm.

### ***Experimental Design***

I tested 13 juvenile frogs (treatments: *pustulosus*,  $N = 6$ ; *petersi*,  $N = 3$ ; noise,  $N = 2$ ; silence,  $N = 2$ ) once each for phonotaxis behavior during the 30 min stimulus presentation period in a dark sound attenuation chamber with a single speaker (Acoustic Systems, Austin, TX; arena dimensions: 1.8 m X 1.1 m). This scenario enabled the frogs to freely behave during exposure to the sound stimuli but precluded precise control of sound pressure levels experienced by animals because they were able to approach or retreat from the broadcasting speaker. When measured with an SPL meter, a 500 Hz tone ranged between 71–95 dB across the arena space. Therefore, subjects in Experiment I might have experienced this range of sound pressure levels.

Animals were filmed under infrared light during the course of the stimulus presentation and the duration of time each frog spent in a 10 cm semicircle surrounding the speaker was recorded. Immediately following the 30 min acoustic presentation the animals were rapidly decapitated and their brains were embedded in TissueTek OCT® embedding medium and flash frozen in liquid nitrogen.

### ***Tissue Preparation***

Brains were maintained in liquid nitrogen for 4–16 d before being transferred to a -20 C freezer where they were maintained until cryosectioning (10–25 d). Coronal sections (16 µm thickness) were thaw-mounted on SuperFrost Plus® slides. Once slides were full they were placed back in -20 C freezer for up to 14 d prior to preparation for *in situ* hybridization.

Brains were sectioned and divided into two alternate series, resulting in at least 32 µm separating any two adjacent sections within a series (see Figure 2a). Only one series was used in this study and all the slides were simultaneously processed for *in situ* hybridization in one batch. Briefly, sections were placed on Superfrost Plus slides (Fisher) and incubated overnight at 65 C with <sup>35</sup>S-labeled ribo-probe specific to *egr-1* (including a sense control) diluted in 1X hybridization solution (Ambion). Non-specific signal was removed with SSC washes. Silver grains densities were visualized by dipping slides in Kodak NTB2 emulsion (VWR), dried at 50 C, then stored in lightproof conditions at 4 C until adequate development of the signal. Emulsion was developed with Kodak D19 developer and fixer (VWR). For a complete description of the *in situ* hybridization procedure see Hoke et al. (2004).

### ***Analysis***

#### ***Phonotaxis***

The duration of time spent in the 10 cm semicircle was converted to percent time in semicircle (duration / 30 min).

#### ***Microphotographs***

Brain sections prepared for radioactive *in situ* hybridization exhibit signal for *egr-1* mRNA as “silver grains,” appearing as black dots on the tissue when viewed with light

microscopy (see Figure 2b). Sections containing the torus semicircularis (midbrain auditory center homologous to the mammalian inferior colliculus) were photographed with a 100X objective under oil immersion. Four toral nuclei (laminar-L, medial-M, principal-P, and ventral-V) were identified for each toral section based on cytoarchitectonic boundaries (see Figure 2a; after Hoke et al. 2004). Within each nucleus a systematic random sampling scheme was followed to ensure an unbiased stereological treatment. For each toral nucleus a random start point within 0–100  $\mu\text{m}$  of the lateral edge was determined by random number generation and this first image was captured. Next, a lateral step distance of 100  $\mu\text{m}$  was taken and a second image captured. This sampling scheme was repeated until the boundary of the nucleus photographed was reached. This lateral to medial step scheme was used for the laminar, principal, and ventral nuclei, while step directions were dorsal to ventral for the vertically aligned medial nucleus. For all animals, 1–10 images were captured for each of the toral nuclei. On each slide, a background image was captured where no tissue was present. This provided a measure of nonspecific background silver grain densities per slide.

The area of each image was standardized at 640 X 480 pixels and cell perimeters and silver grains were outlined with a custom program in Adobe Photoshop® (see Hoke et al. 2004). Cell area and area covered by silver grains positioned within cell perimeters were quantified using NIH ImageJ®. Only silver grains within cell perimeters were quantified. Silver grain densities (ratio of silver grain counts to pixels covered by cells) were calculated for each frame and mean silver grain densities were calculated (subtracting background signal) for each toral nucleus within a subject (ibid.). From these calculations, mean silver grain densities and standard errors were plotted for each treatment group for Experiment 1.

## **Experiment 2: Ontogenetic Trajectories of Phonotaxis in Túngara Frogs**

### ***Animals and Experimental Design***

Between February 2006 and April 2007 I tested 24 male and 18 female frogs throughout their entire postmetamorphic (froglet to adult) development for phonotaxis using a within-subject design at six developmental time points, including three juvenile and three adult time points (Figure 3). Males express a secondary sexual trait (a visible vocal sac) at the adult time points and begin calling while females begin to show external signs of egg maturation. In the field, the smallest adult male collected at a breeding site was 24 mm snout-to-vent length (SVL) and the smallest female was 27 mm (Ryan 1985). Davidson and Hough (1969) found that in the laboratory male túngara frogs are potentially capable of reproduction when they exceed 20 mm SVL.

### ***Development of Conspecific Recognition Behavior***

Subjects ( $N = 42$ ) from a breeding colony at the University of Texas at Austin were collected as larvae from seven broods derived from seven different adult mated pairs and reared until metamorphosis in brood-housed aquaria. During postmetamorphic development animals were housed in the same room as a colony of adults and were thus exposed to conspecific acoustic signals throughout development. Again, such experience has been shown previously to have no effect on vocalizations or phonotactic responses to them (Dawson 2007, Dawson and Ryan 2009). At metamorphic climax (tail fully resorbed at end of Gosner stage 46; Gosner 1960), animals were transferred to terraria where they were group-housed until the first testing time point, and housed individually thereafter and marked with unique toe-clip identifiers. I tested animals at six time points. All subjects could not be tested on a single day, thus the testing for each time point encompassed several days. The six time points included the following in days following

metamorphic climax (mean  $\pm$  SD): (1)  $13 \pm 5$ ; (2)  $51 \pm 4$ ; (3)  $89 \pm 6$ ; (4)  $221 \pm 21$ ; (5)  $388 \pm 22$  and (6)  $400 \pm 21$ . At the final time point (six), the frogs were adults and were injected with human chorionic gonadotropin (hCG), which acts as a ligand for luteinizing hormone receptors, which stimulates the production of gonadal hormones. I used this hormone to ensure that animals at the final testing time point are sexually mature and prepared to mate.

An identical testing procedure (without injection) was conducted on field-collected reproductive male ( $N = 12$ ) and female ( $N = 12$ ) frogs at facilities of the Smithsonian Tropical Research Institute in Gamboa, Panamá in order to compare the behavior of the adult lab-reared animals to that of wild caught adults in which phonotaxis behavior is fully developed (Figure 3); the phonotaxis chambers in Gamboa, Panamá and Austin, Texas are the same dimensions and model (Acoustic Systems).

At each time point, six phonotaxis tests were conducted, including (1) conspecific whine-chuck versus silence, (2) conspecific whine versus silence, (3) an intermediate whine (PE-0.37, see Stimuli below) versus silence, (4) silence versus silence; (5) whine versus intermediate whine (interspecific discrimination) and (6) whine-chuck versus whine (intraspecific discrimination). Recognition was ascribed when animals performed significantly more phonotaxis (choices and association time) in response to the conspecific stimuli (whine and whine-chuck) compared to silence. Subjects were measured for mass and SVL at each time point. Animals were fed fruit flies and pinhead crickets ad libitum three times per week throughout development. For the final adult time point (time point six), all subjects (female and male) were injected intraperitoneally with a 300 International Unit (IU) dose of hCG (Sigma) to induce oviposition in females and ensure that the final time point represented a gravid, reproductive period in which previous studies have demonstrated robust behavior (Lynch et al. 2005b). Using age- and

size-matched frogs in a previous study, I determined that a dosage of 300 IU was effective at stimulating approximately 90% of females to oviposit within 24 hours of injection (Baugh unpublished data). On average, females were tested 19.9 hours ( $\pm 2.1$  hours SD) postinjection and males were tested at 21.0 hours ( $\pm 2.0$  hours SD) postinjection. I recorded the presence and timing of oviposition for all females. Males have not previously been examined for behavioral responses following hCG treatment—I did so here to examine if such effects exist and in order to maintain a symmetrical sex difference study.

### ***Acoustic Discrimination in Juveniles***

In addition to evaluating acoustic recognition, in 2008 I conducted experiments to evaluate acoustic discrimination in juveniles (mean  $\pm$  SD: age postmetamorphic: males:  $31 \pm 17$  days; females:  $26 \pm 13$  days; mean mass  $\pm$  SD: males:  $0.23 \pm 0.057$  g, females:  $0.23 \pm 0.049$  g; mean SVL  $\pm$  SD: males  $13.75 \pm 1.128$  mm; females:  $13.37 \pm 0.899$  mm; approximately time point one and two; see Figure 3). Animals were reared from two unique broods and housed under identical conditions to those used in the 2006–2007 study. I performed three separate two-choice tests: (1) whine versus reverse whine; (2) whine versus intermediate whine; and (3) whine-chuck versus whine. I measured choices by testing 71 froglets across 378 trials, which resulted in 20 froglets that responded in each of the three testing conditions (i.e.,  $N = 60$  choices; 15.9% of trials resulted in a choice).

### ***Preliminary Study on the Effects of hCG in Juveniles***

To examine whether hCG might elevate phonotactic responses in juveniles, a small set ( $N = 13$ ) of juveniles (approximately time point two; mean  $\pm$  SD:  $0.30 \pm 0.09$  g,  $14.08 \pm 2.05$  mm SVL; see Figure 3) were injected with a 150 IU dose of hCG diluted in

0.9% saline and tested approximately 24 hours later in three sequential and identical 15 min two choice trials using the whine versus reverse whine stimuli.

### ***Stimuli***

Five acoustic stimuli were used in this study (see Figure 1a–e). The whine-chuck and whine are natural stimuli recorded in Panamá from a male with call properties centered near the mean for the study population (for spectrograms/oscillograms see stimulus “M” in Ryan and Rand 2003b). These two natural stimuli are identical except that the whine has the chuck component excised. Female and male túngara frogs preferentially approach the whine-chuck stimulus in a whine versus whine-chuck choice test (Ryan 1985). The intermediate whine (PE-0.37) is a synthetic call intermediate between the dominant frequency of the average synthetic túngara whine and the dominant frequency of a related species, *P. enesefae* (Ryan et al. 2003). For this stimulus, seven call parameters were adjusted by the same percentage to synthesize this call as an intermediate between each of the calls of each species. PE-0.37 represents a signal that has an “acoustic distance” that is 37% similar to *P. enesefae* and 63% similar to *P. pustulosus*. For example, PE-0.37 has a fall time of 381 ms, which is 37% different from the *P. pustulosus* fall time (343 ms) and 63% different from *P. enesefae* fall time (446 ms). This linear method was used for the following seven parameters: rise time, fall time, maximum frequency, final frequency, time to one-half the final frequency, time to one-half the peak amplitude and time from the call’s peak amplitude to one-half the peak amplitude during the fall. Female túngara frogs preferentially approach the conspecific whine in a PE-0.37 versus whine choice test (Ryan et al. 2003). The reverse whine is the identical whine stimulus with the temporal order reversed. Field-collected adult females discriminate against this stimulus when paired with the normal whine (Ryan unpublished data). For the white noise stimulus I used the amplitude envelope of the synthetic whine

to shape bandpass filtered white noise (100–5000 Hz passband, the range of frequencies in the conspecific whine; see Ryan et al. 2003).

All stimuli were adjusted to the same peak amplitude and broadcast using CoolEdit 2000 (Syntrillium Software, Scottsdale, AZ, USA) at a sampling rate of 44.1 kHz and 16 bit depth. During all treatments both speakers, including “silent” speakers, were amplified to control for any low frequency sound present in the system. Sound pressure levels of “silent” speakers were inaudible and below the threshold for detection using a GenRad sound pressure level (SPL) meter (model 1982).

### ***Measurements***

As túngara frogs are nocturnal, phonotaxis tests were conducted at night (1800–0200 hours) and filmed under infrared light. All tests were conducted in sound attenuating chambers (Acoustic Systems, Austin, TX; dimensions: 1.8 m X 2.7 m; see Figure 4) with two speakers (Cambridge Audio, Texas; ADS, Panamá) at opposite poles and a ceiling-mounted wide-angle infrared camera (Fuhrmann Diversified, Inc.). Speakers were calibrated at the beginning of the night to 82 dB SPL (re. 20  $\mu$ Pa) at 0.5 m and recalibrated between each animal using a continuous 500 Hz tone and a GenRad SPL meter. In order to measure association time, the chamber was demarcated into equal thirds such that each pole had a zone separated by a neutral center zone. The two terminal one-thirds were subdivided further into equally spaced concentric half circles radiating from the speakers representing the five weighted association zones ( $z_1$ – $z_5$ ). To achieve a fine-grained measure of affiliation, I recorded the time spent in these five zones and multiplied these values by each zone’s weight (proximal to distal distances from the release point: 0.2, 0.4, 0.6, 0.8 and 1.0). Therefore, time spent directly adjacent to a speaker (e.g.,  $z_5$  weight = 1.0) was weighted more heavily than time spent at a more distant location (e.g.,  $z_1$  weight = 0.2). Time spent in the neutral third of the chamber

arena did not contribute to association time. Weighted association time measures have not been used previously in anuran phonotaxis studies; I did so here because it is possible that in juveniles approaching a conspecific signal does not involve a near approach (< 10 cm—the diameter of the choice zone) and could therefore potentially be a more sensitive metric of recognition in young animals. In a similar way, Narins and Capranica (1976) used an approach-distance measurement to obtain finer resolution of auditory behavior in *Eleutherodactylus coqui*.

Each animal at a given time point was tested under all six tests conditions and subjects were tested in a random order without the naïve observer’s knowledge of the animal’s sex during the subadult time points. Test order was assigned randomly for each night of testing and the order was reversed between each frog. Finally, for a given test condition, stimulus position was alternated between the two speakers. There is no evidence that anurans learn to anticipate this alternation sequence, and evidence suggests that short-term memory about preferred stimulus location does not carry over between trials (Ryan unpublished data). Each test began with the subject under a cone in the center of the chamber (release point) while the test stimuli were broadcast for two min. The cone was then raised remotely and the animal was free to behave for 13 min. The summed duration of time spent in the weighted association zones (weighted association time =  $\sum 0.2(T_{z1}) + 0.4(T_{z2}) + 0.6(T_{z3}) + 0.8(T_{z4}) + 1.0(T_{z5})$ , see Figure 4) was recorded as well as choice (ascribed when a subject enters within 10 cm of an active speaker without simply traveling along the perimeter of the chamber). Additionally, I measured latency to enter choice zones, total path length and path lengths within the choice zone (hereafter “locomotor perseverance”; after Hyde and Jerussi 1983). All of these measurements were recorded for behavior at either pole of the chamber, irrespective of whether stimuli were broadcast from both speakers. Animals were very unlikely to approach and make choices

at both speakers—in 1,932 trials there were only 9 trials in which frogs made choices at both speakers within a trial. These occurred in the whine versus PE-0.37 (4 instances), whine versus whine-chuck (2 instances), PE-0.37 versus silence (2 instances) and noise versus silence (1 instance); only one of the nine instances occurred in a female (whine versus whine-chuck in a field-caught adult). Excluding these “double choice” data points from the analyses does not change any of the outcomes of the study as these instances all occurred during time points in which frogs evinced strong choice and association time preferences (one instance at time point three in males, one instance at time point four in males, four instances at time point five in males, one instance at time point six in males and one instance for a field-caught female).

Total path lengths and locomotor perseverance path lengths were measured by tracing each animal’s path in a subsequent viewing of the video-recorded trial (monitor dimensions: 34 X 28 cm) and quantified by placing a grid (1 cm<sup>2</sup>) on the tracing and counting the number of intersections. This value was then converted to actual distances using a conversion factor derived empirically. Prior to data quantification, this grid was tested using a range of path lengths varying from simple to complex and these grid dimensions accurately captured real world path lengths in a manner unbiased by the complexity of the path.

### *Analysis*

#### *Development*

I used repeated measures ANOVAs on choice and weighted association time data to evaluate the onset of species recognition (two within-subject factors: Time point (1–6) and Stimulus (Conspecific and Silence); one between-subject factor: Sex). Due to the relatively infrequent occurrence of phonotactic responses at the subadult time points I

collapsed the results for choice and association time from the six tests into two categories: conspecific (whine and whine-chuck) and silence control (for full descriptive results see Table 1 and 2). Across the six testing conditions there were five opportunities each for subjects to select a conspecific or a silent speaker/zone (Figure 3). Therefore, the ANOVA on the choice results evaluates the number of conspecific (out of five) versus silent choices (out of five) within a subject. Likewise, the ANOVA on the association time results evaluates the weighted association time summed across the five conspecific speakers versus five silent speakers.

Path lengths, latency to choice and locomotor perseverance were also examined with repeated measures ANOVAs (for full descriptive results see Table 1 and 2). Because choice and weighted association time data were positively skewed due to an abundance of zeros at early time points, I performed parameter-free bootstrapped resampling (10,000 simulations/analysis) of repeated measures analyses of variance (R statistical package) to complement the canonical parametric ANOVAs (SPSS 16.0). Statistical *P*-values derived from the bootstrapping approach were in broad agreement with the canonical outcomes. In the instances in which there were minor discrepancies (that did not affect statistical significance), the canonical *P*-values were more conservative than the bootstrapped values and for this reason I report the canonical results here.

#### *Acoustic Discrimination in Juveniles*

I analyzed juvenile choice behavior in the three two-choice discrimination tests with two-tailed binomial and Fisher's exact tests.

#### *Adults*

The effects of hCG were evaluated using an ANOVA for the pre- and postinjection time points within subjects. Interspecific (whine versus PE-0.37) and intraspecific (whine versus whine-chuck) preferences were explored in males and females

before and after hCG treatment. Path lengths, latency to choice, and the proportion of choices made by field-caught adult males and females were examined for sex differences using a student's *t*-test. Additionally, the nonparametric Kolmogorov-Smirnov test was used to compare the distribution of latencies of field-caught males and females. A repeated measures ANOVA was used to assess sex differences in perseverance for field-collected adults.

An alpha criterion of 0.05 was applied to all statistics used in this study and all predictions are two-tailed. All *P*-values for tests involving multiple comparisons were adjusted and are reported after Holm-Bonferroni correction.

## **RESULTS**

### **Experiment 1: Behavioral and Immediate Early Gene Responses to Sound in Juvenile Túngara Frogs**

Behaviorally, froglets spent more time inside the 10 cm choice zone of the conspecific whine compared to the whine of *P. petersi*, noise, and silence (Figure 5a). Neurally, *egr-1* induction was higher in all four subdivisions of the auditory midbrain (torus semicircularis) following stimulation with the conspecific whine compared to the whine of *P. petersi*, noise, and silence (Figure 5b–f). This preliminary result suggests a developmentally early establishment of neural circuits engaged during call recognition (for adults see Hoke et al. 2004, 2005, 2007). No statistical testing was conducted due to small sample sizes in this experiment.

### **Experiment 2: Ontogenetic Trajectories of Phonotaxis in Túngara Frogs**

#### ***Baseline Adult Responses***

Before examining lab-reared developmental time courses using the protocol outlined above, I applied this same protocol to field-collected adults, which are known to

exhibit robust species-typical phonotaxis and therefore could provide some ground-proofing of this method, particularly for the weighted association time measure. Field-collected adults responded in weighted association times as anticipated; females and males spent considerably more time associating with conspecific signals compared to the alternatives (silence, noise, intermediate whine) demonstrating that the protocol described here has the potential to yield reliable information during development (Figure 6). Analyses of these field-collected animals are discussed below (see sections on Adults).

### ***Development Overview***

The lab-reared development study was conducted over the course of more than one year. The body mass and SVL followed an asymptotic growth curve (Figure 7), as predicted for a species with both indeterminate growth and growth rates that decrease with size (Ryan 1985). Froglets from the 2008 discrimination study were within juvenile size ranges (mean  $\pm$  SD: SVL: males:  $13.75 \pm 1.13$  mm; females:  $13.37 \pm 0.90$  mm; mass: males:  $0.23 \pm 0.06$  g; females:  $0.23 \pm 0.05$  g).

The principal behavioral result of this study is that species recognition was present in juvenile frogs at early time points and persisted throughout development, as seen in both of the choice (Figure 8) and association time measures (Figure 9). I observed sex differences in developmental trajectories of locomotor activity, and in particular the locomotor activity directly adjacent to call broadcasting speakers.

### ***Choices***

#### ***Overview***

Field-collected adults made significantly more conspecific compared to silent choices (Wilcoxon Signed Ranks: males:  $Z = 2.83$ ,  $P = 0.005$ ; females:  $Z = 2.95$ ,  $P = 0.003$ ; see Figure 8).

In general for the developmental time series, juveniles (time points 1–3) made conspecific choices in 10.2% of trials and silent choices in 2.4%; adults (time points 4–6), made conspecific choices in 27.8% of trials and silent choices in 4.4% (see Figure 8 and Table 1–2). The repeated measures ANOVA returned significant main effects for the two within-subject factors: Time point ( $F_{5,36} = 13.63$ ,  $P = 1 \times 10^{-6}$ ) and Stimulus ( $F_{1,40} = 62.28$ ,  $P < 1 \times 10^{-6}$ ). The main effect of Sex was not significant ( $F_{1,40} = 0.57$ ,  $P = 0.46$ ). There was a significant Stimulus-by-Time point interaction ( $F_{5,36} = 8.51$ ,  $P = 0.00002$ ); at all six time points frogs made more conspecific choices (C) than silent choices (S) yielding statistically significant responses at time points 2–6, and the strength of this response increased gradually during development (choice count C:S, time point one: 10:2,  $P = 0.06$ ; time point two: 11:3,  $P = 0.049$ ; time point three: 30:7,  $P = 0.003$ ; time point four: 29:8,  $P = 0.04$ ; time point five: 52:11,  $P = 0.00004$ ; time point six: 59:4,  $P < 1 \times 10^{-6}$ ; corrected  $P$ -values). The Stimulus-by-Sex interaction was not significant ( $F_{1,40} = 2.16$ ,  $P = 0.15$ ). Finally, males and females did not differ in the number of conspecific to silence choices at any time point (time point one:  $P = 0.46$ ; time point two:  $P = 0.41$ ; time point three:  $P = 0.91$ ; time point four:  $P = 0.102$ ; time point five:  $P = 0.416$ ; time point six:  $P = 0.095$ ; corrected  $P$ -values). These results show that túngara frogs exhibit positive phonotaxis towards the conspecific signals as juveniles and increasingly perform this species-typical behavior throughout postmetamorphic development.

#### *Individual Differences in Choices*

Figures 10 and 11 depict the extent of between-subject variation in conspecific responsiveness. In Figure 10 I illustrate the number of time points (out of six) in which each individual responded with at least a single conspecific choice. Figure 11 illustrates the total number of conspecific choices performed by each animals summed across all time points and conspecific trials. Both illustrations suggest that while subjects vary in

their responsiveness (e.g., four subjects responded in five out of six time points while one subject never responded), such variation appears not to deviate from a gaussian distribution (Kolmogorov-Smirnov: Figure 10b:  $Z = 1.181$ ,  $P = 0.123$  (two-tailed); Figure 11b:  $Z = 1.141$ ,  $P = 0.15$  (two-tailed)). The extent of variation does, however, reinforce the utility of longitudinal studies in distilling developmental trends, especially for behaviors that are infrequently expressed.

### ***Weighted Association Times***

For field-collected adults, weighted association times for conspecific were greater than silence (Figure 9). The results of the repeated measures ANOVA from choices and weighted association times during development were largely in agreement with each other. As with choices, the main effects of the within-subject factors were significant (Time point:  $F_{5,36} = 20.81$ ,  $P < 1 \times 10^{-6}$ ; Stimulus:  $F_{1,40} = 81.04$ ,  $P < 1 \times 10^{-6}$ ). In contrast to the choice analysis, the main effect of Sex was also significant ( $F_{1,40} = 5.97$ ,  $P = 0.02$ ). As with choices, the Stimulus-by-Time point:  $F_{5,36} = 18.6$ ,  $P < 1 \times 10^{-6}$ . At all six time points frogs associated more strongly with conspecific speakers than silent speakers; this yielded statistically significant responses at time points 3–6, and as with choices the strength of this response increased gradually during development (time point one:  $P = 0.23$ ; time point two:  $P = 0.20$ ; time point three:  $P = 0.004$ ; time point four:  $P = 0.006$ ; time point five:  $P = 0.00001$ ; time point six:  $P < 1 \times 10^{-6}$ ; corrected  $P$ -values). The Stimulus-by-Sex interaction was significant ( $F_{1,40} = 10.32$ ,  $P = 0.003$ ). Finally, males and females did not differentially respond to conspecific compared to silence, except at time points four and five (time point one:  $P = 0.63$ ; time point two:  $P = 0.40$ ; time point three:  $P = 0.28$ ; time point four:  $P = 0.02$ ; time point five:  $P = 0.02$ ; time point six:  $P = 0.82$ ; corrected  $P$ -values). Thus, similar to the choice data these results suggest that túngara frogs exhibit positive phonotaxis towards the conspecific signals as juveniles and

increasingly perform this species-typical behavior throughout postmetamorphic development.

### ***Path Lengths***

Path length analysis was conducted on total path length (total distance traveled (cm) during each 13 min trial) and path length restricted to the choice zone (see Locomotor perseverance below). The analysis of total path length addressed whether there were general locomotor differences during development, both between the sexes and in response to the seven different acoustic conditions, irrespective of whether such locomotor behavior was guided by the presence of stimuli. The ANOVA for total path length returned a significant main effect of Time point ( $F_{5,36} = 13.24$ ,  $P < 1 \times 10^{-6}$ ) and Acoustic Condition ( $F_{5,35} = 2.64$ ,  $P = 0.017$ ) as animals traveled greater distances as they developed (Figure 12). The main effect of Sex was not significant ( $F_{1,40} = 0.003$ ,  $P = 0.98$ ). The Time Point-by-Sex interaction was significant ( $F_{5,36} = 4.06$ ,  $P = 0.002$ ; see Figure 12), as was the interaction between Acoustic Condition-by-Sex ( $F_{5,35} = 4.11$ ,  $P = 0.001$ ) and Time point-by-Acoustic Condition ( $F_{30,11} = 1.85$ ,  $P = 0.004$ ). The three-way interaction, Time point-by-Acoustic Condition-by-Sex, was also significant ( $F_{30,11} = 1.51$ ,  $P = 0.04$ ). With respect to sex, there were not simple sex differences in baseline locomotor behavior, as demonstrated by a lack of difference under the silence versus silence control condition (overall  $F_{1,40} = 0.342$ ,  $P = 0.562$ ; pairwise comparisons: time point one,  $P = 0.98$ ; time point two,  $P = 0.71$ ; time point three,  $P = 0.73$ ; time point four,  $P = 0.25$ ; time point five,  $P = 0.61$ ; time point six,  $P = 0.94$ ). Likewise, there were no sex differences in total path length from the combined acoustic conditions except at time point six ( $P = 0.002$ ) and in field-collected adults ( $F_{1,22} = 9.41$ ,  $P = 0.006$ ) (Figure 12); in both instances this is when females exhibit a large increase due to locomotor perseverance at the choice zone (see Figure 13 and Locomotor Perseverance).

Total path lengths do not allow us to parse out more specific locomotor differences during development and between the sexes. Thus, I also analyzed path lengths inside the 10 cm choice zone around the speaker to examine fine scale patterns of locomotion that could potentially inform our understanding of why adults of both sexes, especially males, perform phonotaxis in the first place.

### ***Locomotor Perseverance***

I refer to movement inside the choice zone as locomotor perseverance because I observed that after an animal enters the choice zone in response to a conspecific signal it restricts most of its movement in the near vicinity of the broadcasting speaker (Hyde and Jerussi 1983). Any locomotor behavior inside the conspecific or silence choice zones was quantified for distance traveled and analyzed with a repeated measures ANOVA and t-tests. Field-collected adult females exhibit perseverance but males do not ( $t_{19} = 4.38$ ;  $P = 0.0003$ ; Figure 13). For the developmental series, perseverance behavior was only exhibited by adult females following hCG injection, resulting in significant main effects and interaction terms from the ANOVA (see Figure 13): Time point ( $F_{5,36} = 24.7$ ,  $P < 1 \times 10^{-6}$ ); Stimulus ( $F_{1,40} = 41.44$ ,  $P < 1 \times 10^{-6}$ ); Sex ( $F_{1,40} = 18.8$ ,  $P = 9.6 \times 10^{-5}$ ); Time point-by-Sex ( $F_{5,36} = 21.6$ ,  $P = 1.2 \times 10^{-5}$ ); Stimulus-by-Sex ( $F_{1,40} = 17.6$ ,  $P = 1.5 \times 10^{-4}$ ); Time point-by-Stimulus ( $F_{5,36} = 24.8$ ,  $P < 1 \times 10^{-6}$ ) and Time point-by-Stimulus-by-Sex ( $F_{5,36} = 22.4$ ,  $P < 1 \times 10^{-6}$ ).

Locomotor perseverance was not exhibited in the silence choice zones and therefore I conducted the ANOVA on perseverance inside the conspecific choice zones only: Time point ( $F_{5,36} = 24.9$ ,  $P = 1 \times 10^{-6}$ ); Sex ( $F_{1,40} = 18.4$ ,  $P = 1.1 \times 10^{-4}$ ) and Time point-by-Sex ( $F_{5,36} = 22.1$ ,  $P < 1 \times 10^{-6}$ ). From Figure 13 it is evident that significance in these analyses is driven by the fact that only reproductive females (time point six) perform perseverance and only in response to conspecific vocalizations; there was a

significant sex difference in locomotor perseverance following choice (subjects not performing at least one conspecific choice were removed) for time point six adults ( $t_{28} = 4.02, P = 0.0004$ ).

### ***Latencies***

Mean latency to choice generally decreased during development. Excluding non-choice trials, latencies to conspecific choice for each time point were as follows (mean, s): time point 1: 513.3; time point 2: 566.1; time point 3: 530.3; time point 4: 414.3; time point 5: 339.4; time point 6: 367.4).

### ***Acoustic Discrimination in Juveniles***

Because juveniles exhibited conspecific recognition early in development, I also performed an independent experiment in 2008 to evaluate the degree of discrimination present. Here, the choice results demonstrated that recognition of the conspecific whine depended on the direction of the frequency sweep as juveniles strongly preferred the conspecific whine when paired with the temporally reversed stimulus (choices whine:reverse whine = 19:1, binomial:  $P = 0.00004$ ). Juveniles also exhibit the species-typical preference for the whine-chuck stimulus to the whine (whine-chuck:whine = 16:4, binomial:  $P = 0.01$ ) and the whine compared to the intermediate whine (whine:intermediate whine = 15:5, binomial:  $P = 0.04$ ). There were no sex differences in responsiveness (of the 20 responding froglets 10 were male and 10 were female) or preferences (Fisher's exact test: whine:reverse whine: males: 10:0, females: 9:1,  $P = 0.99$ ; whine-chuck:whine: males: 9:1, females: 7:3,  $P = 0.58$ ; whine:intermediate whine: males: 8:2, females: 7:3,  $P = 0.99$ ).

### ***Preliminary Study on the Effects of hCG in Juveniles***

In the hCG injected juvenile study, 23.07% of trials (9 out of 39) resulted in a choice. Seven of those nine choices were for the whine and two choices were made for the reverse whine.

### ***Sex Differences in Adults***

Females and males collected in amplexus in Panamá exhibit similar phonotactic readiness at sexual maturity, as demonstrated by similar choice frequency (males: 27 choices [45% of trials]; females: 33 choices [55% of trials];  $t_{22} = 0.86$ ,  $P = 0.39$ ). Despite this fact, the sexes differ markedly in locomotor perseverance in response to conspecific stimuli, with females but not males exhibiting perseverance ( $F_{1,22} = 14.7$ ,  $P < 0.001$ ). This perseverance behavior is not only sex specific but also developmentally specific, occurring only during a period of reproductive competence in adult females. Again, no appreciable perseverance behavior, nor developmental pattern in perseverance behavior, was observed in males (Figure 13). Perseverance thus shows both quantitative and qualitative differences between the sexes and ages.

Latency to conspecific choice in field-collected adults also differs between the sexes, with females having significantly shorter mean latencies (Figure 14;  $t_{57} = 3.27$ ,  $P < 0.01$ ). This difference in latency can also be observed in the distributions, with males exhibiting a positive skew compared to females (Figure 15; Kolmogorov-Smirnov:  $D = 0.366$ ,  $P < 0.05$ ). Also, differences in locomotor perseverance are not simply manifestations of general locomotor differences between the sexes, since path lengths en route to conspecific choice zones did not differ between the sexes ( $t_{22} = 0.58$ ,  $P = 0.57$ ; Figure 16). In a separate study I show that perseverance behavior in females scales positively with the attractiveness of the male call (Baugh and Ryan in preparation (a)).

Finally, sex differences appear to be present in the effects of hCG treatment on weighted association time preferences. It is important to note, however, that I did not design this study to examine the detailed effects of hCG and for this reason did not perform a vehicle control injection or measure steroid hormone levels after injection to ensure that hCG had the expected effects on plasma hormone levels. The following analyses were conducted a posteriori and thus require an element of caution to interpret. Those two caveats aside, hCG did have the intended effect (15 of the 18 females injected with hCG successfully dropped eggs and did so with an average latency after injection of 24 hours ( $\pm 7.1$  hours SD)). And given that similar doses of hCG have been administered to adult female túngara frogs in a previous study using an identical injection protocol (Lynch et al. 2005b), and that neither behavioral differences nor oviposition occurs in vehicle injected females (ibid.), I assume that the same is true in the present study.

An ANOVA for the intraspecific discrimination condition (whine vs. whine-chuck) demonstrated a significant effect of hCG on females (Figure 17); here there was a significant interaction between hormone state (pre/post) and stimulus (whine vs. whine-chuck;  $F_{1,17} = 5.45$ ,  $P = 0.032$ ) wherein the preference for the whine-chuck is evident only in post-hCG females. In males, this same interaction was not significant ( $F_{1,23} = 0.49$ ,  $P = 0.49$ ), although there was a tendency towards a similar pattern of preference for the whine-chuck after hCG treatment (Figure 17).

In the interspecific discrimination condition (whine versus PE-0.37), however, female preferences were unaffected by hCG treatment; there was not a significant interaction between hormone state (pre/post) and stimulus (whine vs. PE-0.37) ( $F_{1,23} = 0.001$ ,  $P = 0.489$ ). In this instance it appears that hCG might increase the strength of the preference but that the preference is there after hCG (main effect of Stimulus:  $F_{1,17} = 9.91$ ,  $P = 0.006$ ; Figure 18). Preferences in males were also unaffected by the hCG treatment in

the interspecific discrimination condition—the ANOVA for males showed no effect for the interaction between hormone state and stimulus ( $F_{1,17} = 0.50$ ,  $P = 0.98$ )—i.e., the preference for the whine stimulus was there before and after hCG (main effect of Stimulus:  $F_{1,23} = 23.4$ ,  $P = 7 \times 10^{-5}$ ; Figure 18).

More generally, the choice results suggested that hCG treatment in females elevated conspecific responsiveness (Figure 19; compare 385 and 402 days), whereas hCG appears to diminish responsiveness in males (Figure 19; compare 391 and 399 days). The bidirectionality of the effect (elevated choices in females and diminished in males) of hCG on conspecific choices for the sexes was significant for the interaction of hormone state and sex ( $F_{1,40} = 6.89$ ,  $P = 0.012$ ). This sex difference was also seen in latencies to choice, wherein females were faster to respond after hCG (mean  $\pm$  SD: post-hCG =  $344.0 \pm 151.8$  s) than before (per subject mean  $\pm$  SD: pre-hCG =  $421.9 \pm 105.2$  s) and males showed the opposite effect (pre-hCG =  $297.6 \pm 155.3$  s; post-hCG =  $415.6 \pm 166.0$  s). The use of vehicle control treatments will need to be used in future studies to confirm that the effects of hCG are not due simply to differences between the sexes in response to handling and injection. However, given that adults were handled and injected 24 hours prior to testing, I think it is unlikely that handling stress explains the sex differences in response to hCG.

### ***Comparison of Lab-Reared and Field-Collected Adults***

Lab-reared, hCG injected (time point six) adult females expressed a similar responsiveness to conspecific stimuli in choice behavior compared to females collected in amplexus in the field (89% versus 92% responders, respectively; Figure 19). Males, however, were less responsive as lab-reared adults than as field-collected adults (58% versus 83% responders; Figure 19). In response to conspecific stimuli (whine versus silence, whine-chuck versus silence, whine versus whine-chuck and whine versus PE-

0.37), the average total path length of lab-reared frogs at time point six (mean  $\pm$  SD: males:  $116.2 \pm 137.9$  cm; females:  $319.2 \pm 247.5$  cm) was approximately half of that observed in field-collected animals (males:  $204.7 \pm 197.5$  cm; females:  $614.4 \pm 440.5$  cm). Males exhibited a decrement in phonotactic choice, association time and total path length after injection of hCG, so I also compared the total path lengths of time point five males (males:  $202.5 \pm 279.6$  cm), which were similar to field-collected males.

An ANOVA of total path lengths pooled across all seven acoustic conditions from field-collected versus time point six males and females returned significant main effects (Lab/Field:  $F_{1,62} = 16.9$ ,  $P = 1.1 \times 10^{-4}$ ; Sex:  $F_{1,62} = 43.2$ ,  $P < 1 \times 10^{-6}$ ) and the interaction between these factors was significant ( $F_{1,62} = 4.9$ ,  $P = 0.03$ ). This was driven by the fact that field-caught females differed from field-caught males more than did their lab-reared counterparts. Because males at time point six (post-hCG) experienced a decrement in phonotaxis, I also substituted time point five males in place of time point six in the ANOVA—this returned similar main effects (Lab/Field:  $F_{1,62} = 7.74$ ,  $P = 0.007$ ; Sex:  $F_{1,62} = 24.26$ ,  $P < 7 \times 10^{-6}$ ) and interaction ( $F_{1,62} = 7.5$ ,  $P = 0.008$ ). These total path length differences between the sexes and localities were due to differences in locomotor perseverance. Males did not exhibit significant perseverance regardless of time point (Lab: time point five:  $28.2 \pm 29.5$  cm; time point six:  $25.5 \pm 28.9$  cm) or locality (mean  $\pm$  SD: Field:  $43.7 \pm 31.3$  cm; Figure 13), whereas females collected from the field performed more perseverance ( $451.6 \pm 292.1$  cm) than lab-reared females at time point six ( $238.0 \pm 185.1$  cm; Figure 13).

### ***Observations from the Field***

I have observed occasionally juvenile túngara frogs at active chorus aggregations in Panamá. In 2007 I collected and tested one field-caught juvenile frog for phonotaxis. I estimated the subject to be approximately two to three weeks postmetamorphic (10 mm

SVL). This subject performed conspecific phonotaxis in each of seven trials (tests included whine versus whine-chuck and whine-chuck versus silence) over the course of four weeks. I held this animal in captivity and dissected it at the end of the field season and determined it to be a male. Two metamorphic froglets (7–8 mm SVL) were also collected in the field and tested once each in whine-chuck versus silence and whine versus whine-chuck trials; both animals failed to exhibit conspecific phonotaxis.

## **DISCUSSION**

Many studies of ontogeny, especially in songbirds and primates, have explored behavioral changes in species-typical signaling (Seyfarth and Cheney 1986, Hauser 1989, Tchernichovski et al. 2001, Rose et al. 2004, Hollén and Manser 2007), including the influence of unlearned forces on the trajectories of song development (Soha and Marler 2001). The present study is unique in that it is one of the most detailed studies of the ontogeny of a sexual behavior in a receiver, and in this case a “wild” animal. Collectively, I have demonstrated that a well-studied behavior—conspecific phonotaxis in frogs—is present well before sexual maturity, but that the full adult form of the behavior for females emerges only at reproductive competence. A gradual increase in the expression of conspecific-directed phonotaxis is mirrored by gradual increases in total locomotion in response to sound. This result supports the “graded” hypothesis of behavioral development. The immediate early gene experiment suggests further that species recognition behavior is made possible through the selective activation of the central auditory system in a manner not unlike that seen in mature adults (Hoke et al. 2004). While species recognition and discrimination are both present in juveniles of both sexes, this result does not necessarily indicate that absence of sex differences in juveniles or between juveniles and adults in the details of their perception of sound. Determining this will likely require a neural approach. A neural technique that does not require

sacrificing the animal would be ideal, as a froglet's sex is indistinguishable during early postmetamorphic growth and a genetic marker for sex in this species, and in almost all anurans, has not been identified (Berset-Brändli et al. 2006).

Although both sexes complete maturity with an overlapping but distinct collection of related behaviors as adults (e.g., females have shorter latencies to phonotactic choice), females also exhibit a nonoverlapping feature—perseverance locomotor behavior. While this particular detail of anuran phonotaxis (or of mate choice in general) has not been reported previously, it is in accord with a longstanding interpretation that females are performing phonotaxis to seek a mate; males, on the other hand, are doing so to join a chorus or simply find a calling location rather than localizing the individual calling (Ryan 1985). In that sense, male phonotaxis might be a form of conspecific cueing—using the presence of conspecifics as an indicator of habitat quality (Keister 1979, Stamps 1988, Stamps 1991).

Young frogs not only approach conspecific signals, they also do so with the same selectivity observed in reproductive adults. The whine versus reverse whine result confirms that juvenile males do not simply approach sound generally or sounds in the frequency range of the conspecific call as the reverse whine has all of the acoustic attributes of the conspecific call except for the direction of the frequency sweep. Likewise, young male and female froglets selectively approach the complex whine-chuck stimulus when paired with the simple whine, demonstrating that the high frequencies contained in the chuck elicit a greater response, presumably due to stimulation of the basilar papilla in the frog's inner ear. Finally, also like adults, froglets prefer the conspecific whine to the intermediate whine, suggesting again a high level of selectivity for an immature animal.

One of the most studied areas of behavioral development is play behavior (Burghardt 2005). Although the premature expression of conspecific phonotaxis described here does not meet the criteria to qualify as play behavior, it has some parallels. Sex differences in the development of play behavior have been demonstrated in diverse groups, including rodents, primates and reptiles. Adaptive explanations for play behavior have been suggested, such as the idea of preparatory development for predation (e.g., object play in felines). Both sexes of túngara frogs must perform phonotaxis as adults, yet the early expression of this behavior during development might signal some form of preparatory development or constraint. As has been suggested in certain instances of play behavior (Williams 1991), if the costs of performing premature phonotaxis in frogs is low enough, the maturation of intermediate behavior will occur if the benefits of having the behavior immediately available for a rapid onset are great. This is a conceivable scenario for seasonal anurans that rely heavily on rainfall patterns and for whom reproductive success is tied so closely with attendance and effort at the breeding site.

### **Function of Juvenile Phonotaxis**

My study of behavioral development has identified a previously unknown behavior—juvenile phonotaxis. While the present study did not examine the potential function of this behavior, it is possible that such behavior has an ecological and adaptive basis. For example, approaching and residing in the vicinity of an active chorus could serve to enable vocal (males) or auditory learning (males and females). I can reject a narrow form of this possibility because sexual behaviors in both sexes (calls of males and phonotaxis in females) are not influenced by acoustic isolation or stimulation during development (Dawson 2007, Dawson and Ryan 2009). Of course, this does not preclude a possible role for auditory learning not evinced through phonotaxis or other forms of auditory learning (e.g., evasive responses to predatory cues).

Another explanation of juvenile phonotaxis is that the behavior functions to maintain natal pond philopatry for developing animals and that choruses provide froglets with an acoustic beacon for their natal pond. In accord, studies have shown that there is no sex bias in dispersal (Marsh et al. 1999, Marsh et al. 2000, Lampert et al. 2003). If froglets disperse from the natal site, phonotaxis to other choruses might cause them to disperse to areas where breeding is likely. In both of these scenarios, juvenile phonotaxis could be a form of conspecific cueing (Keister 1979, Stamps 1988, Stamps 1991, Donahue 2006). Although conspecific cueing might be a function of juvenile phonotaxis, I emphasize this is mere speculation. Conspecific affiliation in juveniles might reflect a developmental precursor to an adult endpoint that subserves this critical behavior in mature animals. I have occasionally identified juveniles that are days to weeks postmetamorphic at chorus aggregations, but have been uncertain of their natal pond origin, and thus whether they were dispersing from or to the chorus.

A final possible interpretation is that this behavior in premature animals is present but not adaptive, yet at the same time is not significantly maladaptive—it is conceivable that this behavior is nonfunctional when expressed prior to sexual maturity. There are a few obvious costs to performing phonotaxis that might suggest there is a function. First, performing phonotaxis requires time and energy. Second, unnecessary movement in the vicinity of a frog chorus could increase predation risk in this predator dense environment.

One important suggestion provided by this study is that the neural circuits responsible for processing and responding in a species-typical way to conspecific advertisement signals might develop long before the coordinated behavior is appreciated. Therefore, when we trace a behavior back to its ontogenetic origins we must not fail to consider antecedent behaviors. Behaviors may appear to develop *de novo* when in fact the underlying neural circuitry has developed gradually. As an example of such

preparation, Bentley and Hoy (1970) demonstrated that the neural network for song generation in crickets is present during postembryonic development and “in place” before the actual sound-producing structures (the forewings) have developed. This network is suppressed until the final moult to adulthood (see also Hoy and Cassaday 1978). Similarly, complex motor patterns such as walking and the righting response in silk moths (*Antheraea pernyi* and *Hyalophora cecropia*) that are typical of posteclosion adults are present in developing pupae but only seen if the pupal cuticle is removed. Shortly before eclosion these behaviors are inhibited and then released from inhibition by the eclosion hormone at the final pupation (Truman 1975). A similar finding was demonstrated in *Antheraea polyphemus*, in which pupae exhibit flight and warm up motor patterns characteristic of adults in the week preceding pupation (Kammer and Rheuben 1974). In a study of auditory and vocal ontogeny in the anabantoid fish *Trichopsis vittata*, Wysocki and Ladich (2001) found that auditory sensitivity preceded vocal capabilities and that vocalizations preceded acoustic communication, as juveniles are not sensitive to the dominant frequencies contained in conspecific sounds. These examples and the present study on frogs point towards ontogenetic precursors to adult behaviors that emerge potentially well before such behavior is demanded of the organisms.

### **Sexual Dimorphism in Adult Response to Gonadotropin**

Besides the dimorphism in the expression adult perseverance locomotor behavior, there is another sexual dimorphism that bears some consideration. At the completion of maturation, both males and females were administered hCG prior to their final phonotaxis tests. This gonadatropin should lead to an increased production of sex steroids in females (primarily estrogen) and males (primarily testosterone and dihydrotestosterone) and should thus promote the expression of sexual behaviors. But

hCG had a dimorphic effect on the sexes, with females experiencing an elevation in responsiveness and males exhibiting a diminution. Again, because a vehicle control was not used and because hormone levels after injection were not measured, I cannot be entirely certain that handling and injection per se were not responsible for the observed differences following hCG injection (see Results).

Lynch et al. (2005b) also showed that hCG administration increased phonotactic responsiveness in female túngara frogs using 500 and 1000 IU dosages and that these dosages resulted in significant increases in plasma estrogen that were similar to the concentrations seen in field-caught amplexant females (Lynch and Wilczynski 2005). In contrast, plasma androgen concentrations in the test females (testosterone and/or dihydrotestosterone) were unaffected by hCG treatment and were similar to that seen in field-caught amplexant females, although there was a tendency towards a decrease in androgen with increasing hCG dosages, which might be due to a relative increase in aromatization of testosterone to estrogen (Lynch and Wilczynski 2005, Lynch et al. 2005b). Here, I show that a 300 IU dosage is sufficient to stimulate female responsiveness and oviposition. Lynch et al. (2005b) showed that a choice preference for conspecific whine was present after 500 or 1000 IU dosages of hCG in two-choice tests when the signal was paired with PE-0.50. Here, I show that for females a preference for a signal of even greater likeness to conspecific (PE-0.37) is present both before and after hCG treatment in association time measurements. Further, I show that the preference for the complex call is present in females only following hCG treatment, despite significant recognition of both conspecific signals before hormonal treatment (Figure 3; time point five); this result is somewhat similar to a finding in midwife toads (*Alytes muletensis*) by Lea et al. (2000) in which females exhibit conspecific phonotaxis after mating yet do not express the species-typical preference for vocalizations of mean (1.8 kHz) over low (1.5

kHz) frequencies. As with the study by Lynch et al. (2005b), I show that latency to choice was similarly reduced after hCG injections in females. Because hCG acts as an agonist for luteinizing hormone receptors, it is possible that hCG directly induced receptive behavior in females. This possibility, however, is unlikely given the results of a study by Kelley (1982), which showed that luteinizing hormone releasing hormone was effective in evoking receptive behavior in female *Xenopus laevis* that were ovariectomized and steroid injected while hCG had no effect. The elevated receptive behavior in females following hCG could be caused by the action of hCG on other hormones such as prostaglandins (Schmidt 1985), a combination of estrogen and progesterone (Kelley 1982) or arginine vasotocin (Schmidt 1985). Again, an important caveat with the hCG component is that I did not conduct a vehicle control treatment. I cannot, therefore, rule out the possibility that the behavioral effects were due to animal handling or injection and not hCG *per se*. This seems a remote possibility, however, given the sudden and striking appearance of locomotor perseverance behavior in hCG injected females, and that the injections occur 24 hours prior to behavioral observations (and thus any effects of injection *per se* should be attenuated) but nonetheless remains a untested possibility. Because hCG injections were used in this study to ensure that the final time point was a reproductively mature time point (and not intended specifically to explore the effects of hCG), I also did not measure plasma hormone levels to ensure that hCG had the expected downstream consequences (elevated estrogen; see Lynch and Wilczynski 2005). Finally, it remains to be seen if phonotaxis can be artificially induced in subadult female frogs (the preliminary experiment described here is inconclusive), but this could potentially help discriminate between direct and indirect effects of hCG if administered to females with immature gonads. With this caveat in mind, the preliminary study of hCG effects in juveniles does not appear to suggest a strong effect of hormone

treatment. A choice frequency of ca. 23% possibly represents a modest increase compared to the approximately 16% frequency observed in juveniles that were not injected; even then, the hCG injected juveniles were slightly larger in size and a few weeks more mature. Therefore, the slightly higher choice frequency might simply be a reflection of slightly more mature animals. Future studies will have to examine this issue further.

I also present the first evidence for the effects of hCG on male phonotaxis. Importantly, the same caveats apply to males as well as females regarding the need for control injections and measuring plasma levels of hormones postinjection. The results appear to suggest that hCG treatment decreases choices and overall movement and increases latencies to choice. In a study by Marler and Ryan (1996), male túngara frogs with higher endogenous testosterone levels had a higher probability of engaging in vocal behavior and application of corticosterone decreased levels of testosterone and the likelihood of calling. In that study the behavior was calling, and not call-seeking, and it is conceivable that these two different responses might be promoted and inhibited, respectively, by androgens. This could be explored by allowing a male frog to choose between engaging in vocalizing or phonotaxis, and manipulating levels of androgens to explore whether such manipulations affect this choice.

## **Conclusion**

Studies of sex differences in anurans have focused on gonadal development (Gramapurohit et al. 2000), the neural expression and hormonal facilitation of sexual behavior in adults (Boyd 1992, Boyd and Moore 1992, Boyd et al. 1992, Boyd 1994), and auditory processing and morphology in adults (Narins and Capranica 1976, McClelland et al. 1997, Mason et al. 2003, Miranda 2007). The topic of ontogenetic changes in behavioral responses to social signals and sex differences therein are entirely unexplored

(Shofner and Feng 1981). Recent studies of the determinants of sexual behavior in adults have demonstrated that sex differences in some species are due largely to differences in the expression of a single gene, such as the *Trpc2* gene in the vomeronasal organ being responsible for male like sexual behavior of mice (Kimchi et al. 2007), and the *fruitless* gene's role in male like sexual behavior and sexual orientation in *Drosophila* (Ryner et al. 1996). There are no such studies with amphibians, but given the sex differences that emerge at reproductive adulthood such an examination would be informative.

Previous authors have suggested that auditory predispositions to conspecific signals in songbirds might function to minimize the learned acquisition of heterospecific vocalizations (Nelson and Marler 1993) or function more generally across vertebrates to guide learned perceptual preferences (Balaban 1997). In túngara frogs it appears that a developmental predisposition to conspecific signals is present, and because vocal and auditory learning are absent in this species (Dawson 2007, Dawson and Ryan 2009) we must consider explanations other than the avoidance of heterospecific vocal learning or more general effects on auditory learning. I suggest that predispositions for conspecific vocalizations are a more general feature of developing vertebrate auditory systems. While a functional explanation for this premature behavior is not available, we must also consider the possibility that phonotactic behavior in premature animals is premature itself; it might not serve a function when first expressed, but its initial expression might be a prerequisite for a normal developmental trajectory.

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## **Chapter 3: Dynamic Mate Choice in Túngara Frogs**

### **ABSTRACT**

Signaling is a dynamic process often occurring over brief timescales, particularly in the acoustic modality. Numerous studies of mate choice and acoustic communication have identified signal parameters essential for species recognition and mate preferences, although these experiments rarely consider the dynamic nature of these processes. Here, I investigate mate choice behavior in female túngara frogs in response to spatially and temporally dynamic presentations of male advertisement calls. My results demonstrate that females are sensitive to the spatial location of preferred call types on a moment-to-moment basis, and that responses are influenced by the continued presence, complexity, sound pressure level, inherent attractiveness of individual male signals, and perceived predation risk (*vis à vis* ambient light levels). In general, my results support the notion that decision-making during mate choice is an open ended process which is sensitive to interruption and persuasion from competing signalers. As for a specific decision rule, I show that for a species in which females actively compare multiple signalers simultaneously, the criterion that underlies the degree of commitment to an initial mate approach is whether there is a state change in the complexity of signals.

### **INTRODUCTION**

There are few decisions in life more important than the selection of a mate. Sexually reproducing animals ensure passage of their genes to the next generation by choosing compatible mates, which often rests on species-specific perception of communication signals. The most significant criterion that must be met when selecting a mate is whether the individual belongs to the same species, as the costs of hybridization

are often significant (Dobzhansky 1951, Mayr 1963). Once a female has classified a pool of courting males as belonging to her species, she typically selects the most attractive member. Although much research has explored what constitutes “attractive” by determining which particular male traits females use to estimate male quality (Lande 1981, Hamilton and Zuk 1982, Bateson 1983, Andersson 1994), the *process* of reproductive decision-making remains relatively unexplored.

The conventional approach to studying mate choice decision-making is to identify whether females employ a sample- or threshold-based decision rule (*sensu* Valone et al. 1996). Sample-based decision rules (e.g., “best of  $n$ ” (Janetos 1980) and “sequential comparison” (Wittenberger 1983)) assume a female makes a decision based on her sampling of available males, whereas threshold-based decision rules (e.g., “fixed threshold” (Janetos 1980) and “one-step” (Janetos 1980)) assume that females select a mate that exceeds some threshold of suitability. This framework has the following disadvantages: (1) these two categories of rules are overly simplified and typically construed as mutually exclusive; (2) for lek-breeding species, in which females by definition compare multiple males simultaneously, a strictly threshold-based decision rule is untenable—females are always sampling multiple males; Valone et al. (1996) suggest that to adequately distinguish between sampling- and threshold-based decision rules requires presenting females with only a single male at a time, yet such a protocol is clearly unnatural for lek species. At the level of the mate choice process, decisions are considered to have two steps (*sensu* Valone et al. 1996). First, sensory information is gathered and used to discriminate between mates. Secondly, a decision rule is engaged to

select the optimal option. Here, I argue that for many species, especially lek-breeders, signaling environments are dynamic and this compels females to iterate the otherwise simplified two-step process mentioned above, and in doing so execute decisions through a process of “temporal updating.”

Temporal updating, or dynamic reproductive decision-making, makes sense for animals that make decisions in social and physical environments that are constantly in flux. In lek-breeding animals, such as many insects, birds, and anurans, females make mate decisions in the midst of temporally variable social signals. Males often gather in high densities to court females using short duration calls that can vary between repetitions in dynamic features such as complexity, amplitude, and call rate—traits that are known in many systems to influence the signal’s attractiveness (Darwin 1871, Andersson 1994, Gerhardt and Huber 2002, Greenfield 2002, Searcy and Nowicki 2005). Given this naturally dynamic acoustic environment, I asked the following question: as a reproductive female compares advertising males, at what point does she make a decision and how does she execute such a choice when the available signals are changing? Hence, rather than attempt to distinguish between two categories of decision rules (that do not necessarily apply to lek species and would require unnaturalistic testing conditions), I take a more psychophysical approach by attempting to gain stimulus control over females such that as an experimenter, I can predict adequately the final mate choice that a female makes. This approach has the added advantage of providing detailed information about the signaling parameters essential for male persuasion during female choice, and therefore makes predictions about optimal behavior in signalers.

Much of the experimental analyses of mate choice document the degree to which females select males based on temporally “static” traits (e.g., male size or plumage coloration) compared to “dynamic” traits (e.g., courtship display vigor). Static traits are relatively invariant within an individual while dynamic traits often vary considerably (Gerhardt et al. 1996, Rosenthal et al. 1996, Hill et al. 1999). In addition to temporal variation in signals, receivers face spatial variation. For example, as receivers approach an acoustic source, the source generally becomes more intense. Interestingly, experimental tests of preferences for both static and dynamic traits are often conducted with static rather than dynamic presentations. Here, I used dynamic presentations in which signals vary in both space and time to test preferences for two dynamic properties of acoustic advertisement signals (amplitude and complexity) to ascertain the fine details of when and how receivers make decisions in variable environments. By doing so I am able to address which specific decision rule explains and predicts commitment during mate choice?

I examined the mate choice behavior of female túngara frogs (*Physalaemus pustulosus*) in response to dynamic playbacks of male advertisement calls by broadcasting two male calls of similar or dissimilar attractiveness and adjusting the two signals as females make an approach. By doing so I addressed the following questions: (1) do females make their final mate decisions before they begin an approach? (2) are females sensitive to moment-to-moment changes in dynamic signaler traits? (3) at what spatial approach distance is an irreversible decision made? (4) which features of acoustic signals influence female decision-making behavior over brief time periods? (5) are

female responses influenced by perceived predation risk? (6) do males also exhibit dynamic phonotactic behavior?

## **The System**

Túngara frogs are small anurans (ca. 30 mm snout-vent length) distributed throughout much of Mesoamerica (Weigt et al. 2005). Males vocally advertise to females during the breeding season (May through December) using a species-typical call, known as the “whine” or simple call (Ryan 1985). Males can ornament the whine with one to seven suffixes known as “chucks” thereby producing what is known as the complex call, or whine-chuck. In nature, females use calls to localize an individual male amongst a chorus and then select a mate by making physical contact, after which the male mounts and clasps the female in a posture known as amplexus. In standardized laboratory two-choice phonotaxis tests, the whine-chuck calls are strongly preferred to whine calls (85% preference strength; Ryan 1985, Ryan and Rand 2003a). Female túngara frogs also exhibit strong preferences for calls of higher amplitude over lower amplitude alternatives, which presumably results in attraction towards nearer males and thus reduces travel time (Ryan and Rand 1990). While females prefer complex to simple calls, for a given complex call (natural or synthetic) they do not appear to prefer a more complex version (e.g., whine with 4 chucks) to a less complex version (e.g., whine with 2 chucks) (Ryan unpublished data). This study addresses shows that it is possible that such a preference is present but that it is exhibited under different testing conditions.

Individual male túngara frogs differ in the attractiveness of their calls. Ryan and Rand (2003b) studied female mate choice in response to recorded natural calls from males in the study population (Gamboa, Panamá) and showed that the complex calls of some males are consistently more attractive than those of others. Here, I used this natural

variation in call attractiveness to examine how such intrinsic differences influence the extent to which females commit to an initial approach in the face of dynamic changes in call complexity.

During the breeding season, females encounter a range of environmental conditions at the chorus, including variable ambient light conditions depending on the lunar cycle and cloud cover. An earlier study in túngara frogs showed that females are sensitive to light levels in static mate choice tests but, paradoxically, found that females were less likely to choose a nearer male under dim light conditions compared to darkness (Rand et al. 1997). I address this apparent paradox by performing dynamic choice tests in which stimuli are matched for intensity, and are thus not perceived as differing in proximity, and yet differ dynamically in complexity; I predict the frequency of reversed mate choices will decrease if females perceive dim light conditions as more risky because reversing incurs more time in motion and therefore presumably greater conspicuousness. This result would suggest females are weighting preferences for signal complexity against the costs (including time, energy, and conspicuousness) associated with greater movement.

Phonotaxis in male anurans is examined less frequently and it is assumed that in chorus breeding species (e.g., túngara frogs) this behavior functions to guide males to aggregations for the selection of a calling site and in territorial species to repel rival male intruders (Hödl et al. 2004). Under static playback conditions, male túngara frogs exhibit phonotaxis towards the conspecific advertisement signals and, as with females, prefer the complex call with similar preference strengths (see Chapter 2; Bernal et al. in press (a), Baugh and Ryan in review (a)). In this study I performed a dynamic phonotaxis experiment with males to determine if they exhibited updating behavior similar to that of females. In females, selection of the complex call is an expression of mate preferences—

in males, such behavior might increase the likelihood of selecting a high quality calling site because complex calls are indicative of high density choruses which confer increased per capita mating success for males and lower predation risk (Ryan et al. 1981, Bernal et al. 2007).

## **METHODS**

Here I explain the general methods that all experiments share in common. In the Results I explain each experiment individually and describe the results for each inline. Reversal and latency results are summarized in Tables 3 and 4, respectively.

### **Animals**

I conducted all experiments during the breeding season between the months of June and September in 2007 and 2008 at facilities for the Smithsonian Tropical Research Institute in Gamboa, Panamá (9°07.0'N, 79°41.9'W). I collected frogs as amplexant mated pairs from breeding aggregations between 1900 and 2200 h and performed behavioral testing between 2000 and 0500 h. Animals were held in small plastic bags in dark, quiet conditions before testing. To prevent resampling I marked individuals with a unique toe clip combination, measured the mass and snout-vent length and returned them to their original site of collection within 12 h. In marking frogs, I followed the Guidelines for the Use of Live Amphibians and Reptiles in Field Research, compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR). In total, 422 subjects (375 females and 47 males) were tested in 1,400 trials, resulting in 1,062 successful choices (i.e., 75.9% of trials resulted in choices).

## Stimuli and Experimental Design

In total, I performed 21 different phonotaxis experiments of which 20 involved females and one (*experiment t*) involved males. I used nine stimuli throughout this study (Figure 20) and all playbacks were done using a two-choice design. All nine stimuli were matched for the peak amplitude of the whine before playback and broadcast from small speakers located at the center of the two poles of an arena (Figure 21). I used the following three synthetic stimuli: a simple whine (W), a whine with one chuck (Wc), and a whine with three chucks (W3c). The W3c stimulus simply has the chuck from Wc in triplicate with a 4 ms inter-chuck interval. The chuck on these two complex calls (Wc and W3c) is twice the peak amplitude of the whine. The whines in these signals consist of only the fundamental frequency; it has been shown previously that the upper harmonics of the whine do not influence mate choice in the laboratory (Ryan and Rand 1990, Rand et al. 1992), and that these synthetic calls are as attractive as natural calls (Ryan unpublished data). These stimuli were synthesized based on the mean values for the fifteen parameters of the calls in the population by shaping sine waves using custom software (J. Schwartz, Pace University; sample rate 20 kHz and 8 bit). The mean values for the population were calculated based on the calls from 50 males recorded in July 1996 with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003b). The six remaining stimuli were natural advertisement calls recorded from three males (a whine-chuck was recorded from each male and the chuck was digitally excised to leave just the whine) in Gamboa, Panamá. These stimuli have been used previously to explore female preferences in the Gamboa túngara population. The results from that study (Ryan and Rand 2003b) demonstrated that calls Wc (n1) and Wc (n3) are equivalently attractive, while Wc (n2) is

significantly more attractive than Wc (n1) (see caption in Table 3 for stimulus naming conventions). In a few experiments (*k*, *l*, *m*, and *n*) I used these natural differences in attractiveness as leverage for examining the role of inherent male call qualities in influencing female commitment. A female that continued to approach the same speaker after the stimulus switch was considered “committed.”

Frogs were tested under infrared light in a sound-attenuating chamber (Acoustic Systems, Austin, Tx) measuring 2.7 X 1.8 X 1.78 m. Before each subject was tested, I calibrated both speakers to 82 dB SPL (re. 20  $\mu$ Pa) at the center of the arena (1.35 m from each speaker) using the W stimulus (GenRad 1982 SPL meter). Each trial began with the subject placed under a cone at the center of the acoustic chamber (i.e., release point; Figure 21) for three minutes while the two stimuli were broadcast antiphonally at a rate of one call per two seconds from each of two ADS L210 speakers opposite one another. The cone was then lifted remotely and the phonotactic behavior was monitored via a ceiling-mounted infrared camera and television monitor outside the chamber. The chamber was divided symmetrically by boundaries (hereafter “switch boundaries”) at given distances from the speakers (dashed lines, Figure 21). These dashed lines were visible only to the human observer (outlined by transparencies on the monitor). For most of the experiments one of the two speakers initially broadcast a preferred stimulus (this “target” speaker was selected randomly and then alternated between tests and females to minimize any potential side bias; see Results) while the opposite speaker broadcast the less preferred call of the pair. When the subject crossed the switch boundary nearest the preferred stimulus (as measured from the face of the speaker), the human observer pressed a key on the playback computer’s keyboard which initiated a custom program in the acoustic software program Signal®; these programs introduced a 500 ms delay to prevent the premature occlusion of a stimulus, and then each program performed a

custom operation suited to the specific question addressed in the given experiment. For many of the experiments the two stimuli were switched and the amplitude of the distant stimulus (the one that was not initially approached) was amplified by a factor that equilibrated the mean peak amplitude along the switch boundary (this was empirically determined). I describe these experiments in the Results section in the same order as they are found in Table 3. In each experiment the same three behaviors were scored and a fourth one was calculated: (1) does the subject make a choice and if so, does it involve a reversal or rather a choice for the initially approached speaker (a non-reversal)? (2) latency to the switch boundary (time lapsed between lifting the cone and crossing the switch boundary nearest the target speaker), (3) overall latency to choice (total time lapsed between lifting the cone and the choice), and (4) the latency to choice following stimulus switching (overall latency minus boundary latency).

I scored a phonotactic choice when a frog approached one of the speakers within a 10 cm radius without simply following the wall. A frog failed to exhibit a phonotactic choice if it was motionless for the initial 5 min after the cone was raised or during any 2 min interval thereafter, or if the animal failed to make a choice within 15 min after the cone was raised. If a frog failed to approach the preferred stimulus in a pair (complex call) in the experiments involving initially a simple versus complex call (all experiments except *g*, *h*, *i*, and *j*) and instead approached and selected the less preferred stimulus (simple call) these responses were scored as a “no response” along with those trials in which a frog simply made no choice. In these instances frogs were re-tested, in which case they typically approached the preferred stimulus (approaching the preferred stimulus occurred ca. 85% of the time).

I tested each female twice in the same condition (2 replicates) and used the nonparametric Mann-Whitney U test (two-tailed) to compare the number of reversals

across treatments (females could thus reverse 0, 1 or 2 times total within a test condition). To ensure independence, I used a unique pool of females in all the experiments in which I made pairwise comparisons. An alpha criterion of 0.05 was applied to all statistical tests. Additionally I used paired t-tests to examine differences in female latencies (latency to switch boundary, latency to choice following stimulus switching, and overall latency to choice) between trials in which a given female reversed choice compared to the identical test condition in which she did not reverse. In order to achieve a sufficient sample size to examine these latency differences between reversals and non-reversals within females (i.e., females that reversed on one of the two trials only) I used data from *experiments b, k, l, m, and n* (i.e., all the experiments that involved a simple versus complex call followed by stimulus switching at the 20 body lengths boundary and a boost of 2 dB).

To ensure that testing females on sequential trials did not influence their behavior, I performed several analyses on reversal behavior and overall latency to choice in the largest single experiment (*experiment b*), including (1) a paired t-test of overall latency to choice between the first and second trials for females that reversed on zero out of the two trials, and (2) a paired t-test of overall latency to choice between the first and second trials for females that reversed on two out of the two trials. It would not be informative to perform an analysis of latency by trial number for females reversing on one of the two trials since latencies are consistently higher when females reverse choices (see Results). I extended this latency analysis across all experiments by performing a repeated measures ANOVAs on the overall latency from the two replicates for females that made zero reversals and for females that made two reversals (one within-subject factor (trial number) and one between-subject factor (experiment type)). Finally, to ensure that testing females in two back-to-back trials did not influence the probability of reversal, I performed a binomial exact test on reversal/non-reversal results for trials 1 and 2.

## RESULTS

To identify any potential for side bias in the acoustic chamber, I performed trials ( $N = 235$ ) in which both speakers broadcast the identical standard call (W versus W or Wc versus Wc). I found no evidence of a side bias in either 2007 or 2008 (left:right choices: 122:113;  $P = 0.60$ ).

### Reversals

#### *Experiments a–g: effects of complexity and amplitude across space using synthetic signals*

In *experiment a* I initially broadcast the synthetic whine versus whine-chuck, switched the stimuli after the female crossed the switch boundary towards the whine-chuck (75 cm from the face from the speaker, ca. 20 body lengths from the Release Point), and then rebroadcast the stimuli without any amplitude change (i.e., the near stimulus, which has become the whine, is approximately 2 dB louder due to proximity). Then I recorded if the female continued on her original trajectory and selected what is now the whine, or if she reversed course and selected the distant stimulus (whine-chuck) behind her. In this condition females reversed directions and selected the distant whine-chuck in 30% of trials, and therefore maintained their original trajectory selecting the near whine for the remaining 70% of trials. Females in *experiment b*, in which the distant whine-chuck stimulus was amplified an additional 2 dB after switching (thus equilibrating the peak amplitude of the two options at the switch boundary), reversed significantly more frequently (44.3%) than females in *experiment a* ( $U = 1080$ ,  $P = 0.031$ ). *Experiment b* was used as a standard to compare several of the other conditions in this study; I performed 10 planned comparisons against *experiment b* using 11 independent groups of frogs and thus did not correct for multiple comparisons (number of comparisons  $\leq k-1$ ); additionally, I employed a large sample size (70 females) evaluated

across two summer field seasons to provide statistical power and to minimize the likelihood of an unusual sample.

In the control *experiment c* all the manipulations in *b* are present except that the stimuli are simply rebroadcast from the original locations after the 500 ms delay. In this condition, females reversed in 0% of the trials, suggesting that at a minimum of 75 cm from the approached speaker a female's decision is immutable provided her options do not change. This condition also confirms that the brief interruption of the playback program does not itself interfere with the decision-making process. The reversal frequency in this control condition differed significantly from *experiments a* ( $U = 220, P = 0.000455$ ) and *b* ( $U = 210, P < 10^{-6}$ ).

In *experiment d* I moved the switch boundary farther from the release point (60 cm from the face of the target speaker, ca. 25 body lengths from the Release Point) and compensated for the distance by boosting the distant stimulus by 6 dB when the female crossed the switch boundary. Using the synthetic W versus Wc stimuli again, I found that the reversal frequency (42.5%) did not differ from *experiment b* ( $U = 678.5, P = 0.82$ ). *Experiment e* was identical except the switch boundary was 100 cm (ca. 10 body lengths from the Release Point) from the face of the target speaker, and I boosted the distant stimulus by 1.33 dB to compensate. In *e* I found a reversal frequency of 65.0%, which was significantly greater than the reversal frequency seen in *b* ( $U = 481, P = 0.020$ ) and marginally different from *d* ( $U = 134, P = 0.054$ ). In *experiment f* I maintained the switch boundary at 20 body lengths but instead of boosting the distant Wc by 2 dB as in *b*, I boosted it by 4 dB to examine if reversal frequencies would increase beyond the 44.3% seen in *b*. This was not the case; reversal frequencies remained approximately the same as in *b* (45.0%;  $U = 697, P = 0.975$ ).

In *experiment g* I initially broadcast W versus Wc but when the female crossed the switch boundary nearest the Wc, I changed it to a W while leaving the distant signal a W and amplifying it 2 dB to compensate for the distance. Here, I found a significant decrease in reversal frequency (12.5%) compared to *experiment b* ( $U = 352, P = 0.0002$ ) but also a significant increase in reversal frequency over *experiment c* ( $U = 150, P = 0.018$ ).

***Experiments h–k: adding and subtracting complexity to initially equivalent calls***

In *experiment h* I initially broadcast the synthetic Wc from both speakers and then subtracted the chuck from the approached speaker after the female crossed the switch boundary (20 body lengths from the Release Point). Therefore, this experiment had different initial conditions but identical conditions at the switch boundary compared to *experiment b*. Here, I found a reversal frequency of 37.5%, which did not differ significantly from *experiment b* ( $U = 621.5, P = 0.408$ ). However, in the control condition, *experiment i*, which was identical to *h* except the chuck was not removed, there was a decline in reversal frequency (10.0%) that was significantly different than that seen in *experiment h* ( $U = 122, P = 0.014$ ). Similarly, in *experiment j*, in which I initially broadcast the synthetic W from each speaker and then added the chuck to the initially unapproached speaker once the female crossed the switch boundary (20 body lengths), I found a reversal frequency of 52.5%, which does not differ from *experiment b* ( $U = 615.5, P = 0.371$ ), but does differ from the control version (7.5% reversals in *experiment k*) of this experiment ( $U = 71, P = 0.00009$ ).

***Experiments l–o: manipulating complexity using natural signals that vary in attractiveness***

Here I used natural signals that varied in attractiveness to examine if such inherent qualities influence reversal frequency in a predictable manner. First, however, I

demonstrated that the complex versions of these natural calls were indeed preferred to their simple versions. This was a necessary step because there are some males (30% of males; Baugh and Ryan in preparation (b)) for which a male's complex call is not preferred to his simple call. For the three male's natural calls used here in conventional (i.e., static) mate choice experiments, the complex version was indeed preferred to the simple version (Wc(n3) versus W(n3): 16:4, binomial (two-tailed):  $P = 0.011$ ; Wc(n2) versus W(n2): 16:4, binomial (two-tailed):  $P = 0.011$ ; Wc(n1) versus W(n1): 18:2, binomial (two-tailed):  $P = 0.0004$ ).

For the dynamic experiments I initially broadcast a simple natural whine versus a different male's complex whine-chuck and then subtracted that chuck once the female crossed the switch boundary nearest it and simultaneously added the distant male's chuck to his own whine. In *experiment l* and *m* I used natural calls n1 and n3, which are known to not differ in attractiveness. *Experiment l* began with the n3 male using a complex call, whereas in *experiment m* I symmetrically began with the n1 male as complex. Reversal frequencies for *experiments l* (30.0%) and *m* (38.3) did not differ ( $U = 410$ ,  $P = 0.518$ ). In *experiments m* and *o* the two natural calls (n1 and n2) do differ in attractiveness with n2 significantly preferred in standard phonotaxis tests. In a comparison of these conditions it did matter which signal began as complex. When the more preferred call was also the initially complex call (*experiment n*), reversal frequencies were low (28.3%) compared to the reciprocal condition (*experiment o*, 63.3%) and these reversal frequencies differed significantly ( $U = 247$ ,  $P = 0.001$ ).

***Experiments p–q: do multiple chucks elicit greater commitment from females?***

*Experiment p* was identical to *b* except I used a synthetic whine followed by three chucks for the complex call. I found a marginally significant decrease in reversal frequency (30.0% compared to 44.3% in *b*) in this condition ( $U = 820$ ,  $P = 0.057$ ). I

repeated this experiment in *q* but used 40 females each tested once rather than 20 females tested twice and found a similar result (27.5%), suggesting that this decrease is real, and given the similarity of the reversal frequencies between *p* and *q* it appears that testing females more than once did not influence the outcome of these experiments (see *Latencies* below for more on this topic). Thus it appears that multiple chucks do elicit greater commitment to an initial approach compared to single chuck calls.

#### ***Experiments r–s: manipulating call presence/absence***

In *experiment r* I initially broadcast the synthetic W and Wc but then I simply ceased broadcasting the Wc once the female crossed the switch boundary nearest it. In this condition, females reversed 100% of the time and chose the distant W. In *experiment s* I ceased both speakers and found that 0% of females reversed but that 25% of females continued on their original trajectory and selected the near, but now silent, speaker. The remaining 75% of females did not make a choice.

#### ***Experiment t: male reversals***

*Experiment t* was identical to *experiment b*, but tested males rather than females. Here, I found that males exhibited this reversal behavior with a frequency (35%) that does not differ from that of females in this condition ( $U = 597$ ,  $P = 0.276$ ). However, many more males than females were disqualified during these trials due to an unwillingness to continue phonotaxis following stimulus switching (see Table 3).

#### ***Experiment u: the effect of perceived predation risk on reversals***

*Experiment u* was identical to *b* except I placed a dim ambient source of light (GE brand night light model no. 55507; Fairfield, CT, USA) on the ceiling of the chamber that had spectral properties within the range of variation for natural moonlight (with a broad peak around 510 nm; Taylor et al. 2008). I found a significant decrease in the reversal

frequency (20.0%) compared with *experiment b* ( $U = 483$ ,  $P = 0.005$ ). The effect of the light was specific to reversibility and not preferences generally. In only 9% of trials did females approach and choose the whine (and therefore there was no stimulus switching) and this is within the normal range of preference under dark conditions (ca. 15%). In other words, the light source did not affect preferences generally but rather specifically by reducing the reversal frequency and consequently travel time and movement.

### **Latencies**

Table 4 outlines the mean latency to the switch boundary, latency to choice after stimulus switching, and overall latency to choice with data pooled from the basic experiments involving a simple versus complex call and stimulus switching with a 2 dB boost (*experiments b, l, m, n, and o*). From these experiments I selected females that reversed on one of the two trials and performed paired t-tests to determine if these three latency measures differed for trials involving a reversal compared to those that involved a non-reversal. I found that the latency to the switch boundary did not differ between reversal and non-reversal trials ( $P = 0.649$ ). The latency to choice following stimulus switching, however, differed significantly for reversals and non-reversals due to the longer distance that females traveled during a reversal ( $P < 10^{-6}$ ). Finally, the latency to overall choice differed significantly between reversal and non-reversal trials ( $P = 0.00002$ ).

I tested each female twice in these experiments (trial 1, trial 2). To ascertain whether female behavior was affected by trial number, I analyzed overall latency to choice for the first and second trials, comparing these latencies within subjects for females that reversed on both trials and females that reversed on neither trial from *experiment b*—my single largest experiment (again, I did not compare latencies across the two trials for females performing 1 reversal and 1 non-reversal because latencies

differ significantly depending on whether a reversal occurs). In both instances, latency to choice was unaffected by trial number (paired t-test: zero reversal females:  $t = 1.43$ ,  $df = 20$ ,  $P = 0.168$ ; two reversal females:  $t = 1.09$ ,  $df = 12$ ,  $P = 0.295$ ). To extend this analysis to all the experiments I used repeated measures ANOVAs for females that reversed on zero out of two trials and females that reversed on two out of two trials (1 within-subject factor: trial number; 1 between-subject factor: experiment type). In both analyses the main effect of trial number was not significant (zero reversals:  $F_1 = 1.56$ ,  $P = 0.213$ ; two reversals:  $F_1 = 1.28$ ,  $P = 0.260$ ), nor was the interaction term for either analysis significant (zero reversals:  $F_{19} = 1.00$ ,  $P = 0.453$ ; two reversals:  $F_{15} = 1.14$ ,  $P = 0.329$ ). To address whether females were more or less likely to reverse on the first or second trial I used an exact binomial test (two-tailed) on the number of reversals performed on the first versus second trial for females making one out of two reversals and found no effect in *experiment b* ( $P = 0.07$ ) or across all experiments ( $P = 0.14$ ). In sum, I conclude that testing females twice does not influence latency or reversal behavior and is an acceptable method of data acquisition.

Males tended to have slower approaches than females for both non-reversal trials (Mean  $\pm$  SEM (s): boundary latency:  $128.5 \pm 16.6$ ; choice after switch latency:  $41.19 \pm 5.43$ ; overall choice latency:  $169.7 \pm 19.3$ ; compare to values in Table 4) and reversal trials (boundary latency:  $143.1 \pm 22.2$ ; choice after switch latency:  $186.9 \pm 35.9$ ; overall choice latency:  $329.9 \pm 46.7$ ; compare to values in Table 4).

Lastly, under dim light females moved faster and consequently had shorter latencies to overall choice compared to darkness (*experiment b*) when overall latencies were compared for females performing both non-reversal choices (mean (s)  $\pm$  SD: dim light:  $106.7 \pm 98.5$ ; dark:  $162.2 \pm 131.2$ ) and reversal choices (dim light:  $127.2 \pm 77.6$ ; dark:  $163.8 \pm 121.1$ ).

## DISCUSSION

Mate selection in túngara frogs provides a compelling model for examining the details of auditory behavior, including temporal aspects of the mate choice process. The simplicity of conditions used in many studies of mate choice precludes a thorough appreciation of the variation in preferences that exist—this limits our understanding of the strength and direction of sexual selection. My results demonstrate the following: (1) frogs are sensitive to the spatial position of a preferred call and in many instances the reactions to altered calls are instantaneous (see online electronic material); (2) decisions are not always finalized prior to an approach—choices are flexible and depend on a consistent repetition of uninterrupted complex calls; (3) a female's commitment to an initial choice increases as distance to the target decreases; or alternatively, a female's commitment may increase as the distance she has traveled increases (i.e., her locomotor investment); (4) the complexity, amplitude, and intrinsic attractiveness of signals influence the probability of reversing phonotaxis toward an alternative male; (iv) a non-acoustic condition, ambient light levels, influences reversal frequency and choice latency; (5) temporal updating during phonotactic behavior is not limited to females, as males also exhibit this flexibility.

I can now answer the question proposed at the beginning of this study regarding a specific decision rule that explains commitment during mate choice. Five of the experiments addressed this general question (*experiments c, g, i, k and s*). Collectively interpreting these results, I deduce that females are strongly committed to an initial choice provided there is no change in signal complexity that leads to a simple versus complex contrast between the two sources, but that when there is such a change (*experiments a, b, d, e, f, h, and j*) females update such information and change their mate choice decision between 30–65% of the time.

A few other studies have pursued dynamic mate choice in anurans; I do not know of other taxa in which similar experiments have been conducted. In reed frogs (*Hyperolius marmoratus*), Dyson et al. (1994) found that females will reverse course after initially approaching (one body length) a preferred stimulus (leading call) if the stimuli are switched. Likewise, Gerhardt et al. (1996) tested the preference strength for pulse number in *Hyla versicolor* and demonstrated that females, after initially approaching a high pulse number call, would reverse directions if this preferred stimulus were suddenly switched with a less preferred low pulse number alternative. By varying the pulse number of competing calls across experiments within the natural range of variation, and adjusting the distance and amplitude of the sources, Gerhardt et al. (1996) obtained reversal frequencies that averaged about 50%, and peaked at approximately 75% under conditions of greatest signal difference. These previous studies provide evidence that in at least a few species of frogs decision-making involves temporal updating. The extent to which this type of behavior is influenced by other signal parameters and environmental conditions is explored further here, as well as an estimate of the cost of temporal updating (latency to choice and distance traveled increase during reversals).

From previous research in túngara frogs it was clear that females prefer the complex call. My study shows that this preference hinges critically on the timing of complex call production—females prefer males that are presently producing a complex call. All males are capable of producing complex calls but choose to do so depending on the social environment, with complex calls more commonly produced during bouts of vocal competition between neighboring males (Bernal et al. in press (b)); in the presence of females (Akre pers comm); when predation risk is perceived to be lower (Tuttle et al. 1982, Jennions and Backwell 1992, Phelps et al. 2007); and when injected with arginine vasotocin, a neuropeptide known to modulate social behavior in a number of species

(Moore and Miller 1983, Boyd 1994, Chu et al. 1998, Marler et al. 1999, Sanantgelo and Bass 2006), including túngara frogs (Kime et al. 2007). I show that the continued production of complex calls is critical when females are assessing and approaching potential mates and therefore males should be under selection to maintain complexity during peak female attendance at the chorus. Furthermore, males would benefit by using available cues correlated with female presence to inform their short-term decisions about which type of call to produce and maintain. Males might be privy to information about female proximity from visual, olfactory, acoustic, or tactile (especially water surface vibrations) cues (Akre pers comm). This potential interaction between signaler and receiver over small spatial and temporal scales is presently unexplored but could yield important insights into short-range animal communication, an area recently receiving attention in other systems (Cator et al. 2009) and of considerable promise in examining the validity of a predominant view that suggests behavioral plasticity is limited to certain taxonomic groups and tasks (Krams 2001, Naguib et al. 2008). Further, because environmental transmission of the chuck is weaker than the whine (Ryan 1986), this might provide what could be considerable pressure on males to selectively use complex calls when females are likely to be listening.

A study by Márquez et al. (2008) examined the related issue of mate choice as a function of call amplitude, using a treadmill to derive another metric of preference. In addition to investigating the role of amplitude, I suggest that the approach taken in the present study also provides a window into the dynamics of the decision-making process. I show that the reversibility of a particular decision can be titrated spatially, wherein females are more likely to maintain their present trajectory the closer they approach their target. The present study used distances of 10, 20 and 25 body lengths to demonstrate how the likelihood of a reversal decreases as the spatial investment increases and/or

distance to the source decreases. Collectively the dynamic studies of mate choice by Dyson et al. (1994), Gerhardt et al. (1996), Marquez et al (2008) and the present study decompose this decision-making process into spatial and temporal domains and in doing so demonstrate convincingly that anuran behavior is more flexible than previously appreciated and that this dynamic mate choice paradigm could be applied to other systems wherein signalers simultaneously compete for receivers (e.g., acoustic signaling in crickets, multimodal signaling in manakins). This study also motivates new research on the mechanisms of attention that underlie this behavior and the potential hormonal modulation of the neurotransmitter systems in the brain (e.g., catecholamines) that might serve as the basis for temporal updating. For example, a female túngara frog's hormonal state can influence some general aspects of her mate choice phenotype (Lynch et al. 2005a, Lynch et al. 2005b), but how might her internal milieu constrain or promote temporal updating of information in mate choice within a reproductive bout and between bouts during the breeding season? For example, by injecting reproductive females with either agonists or antagonists to the neurotransmitter dopamine it might be possible to modulate the extent of temporal updating. Doing such manipulations across the reproductive cycle would illuminate how circulating gonadal steroid hormones interact with the dopamine systems in the brain to yield temporal updating behavior.

As mentioned previously, a continuous acoustic presence is essential for male success during female assessment. The call cessation experiments represent a reasonably natural scenario with which to examine this—multiple females at a chorus might be assessing the same male and once the first female selects a given male, he is out of the pool of potential mates and his calling ceases abruptly. For other females nearby, continuing an approach after cessation would be of no value. Males also tend to cease calling when they detect predators (Tuttle et al. 1982, Jennions and Backwell 1992,

Phelps et al. 2007) and therefore it would be beneficial for females to restrict movement following call cessation. For many anuran species (e.g., treefrogs, spring peepers), including túngara frogs, the single best predictor of male mating success is time spent at the chorus—males who call more achieve more matings and therefore there are strong costs and benefits to persistent calling (Ryan 1985). From my call cessation experiment (*experiment s*) it appears that some females, or all females some of the time (25%), have indeed committed to an initial decision and even call cessation fails to dissuade their choice or arrest their movement. Future studies will explore the extent to which individual variation in females provides an explanation for these results. Differences between reversal and non-reversal trials, however, do not appear simply to be explained by motivational differences because the latency to the switch boundary (a proxy for motivation) did not differ between reversal and non-reversal trials. For fickle females, the few moments before executing mate choice by physically contacting a male might be the crucial time window for male persuasion. This study demonstrates that such a time window exists and is perhaps narrower than previously thought (Schwartz et al. 2004).

I also showed that ambient light levels, which elevate perceived predation risk (Rand et al. 1997), also influence reversibility, suggesting again that multiple parameters play a role in determining reproductive decision-making; this result further supports the idea that reproductive behavior in anurans is not simply a stereotypic response elicited by a given stimulus (e.g., advertisement signal), but rather an context-dependent array of responses.

I suggest that mate choice behavior in túngara frogs is not simply a two-step process of evaluating signals and applying a decision rule, as it is commonly framed in behavioral ecology (Valone et al. 1996); such conceptualizations ignore the role of executing the decision, including the iterative process of temporal updating during

choice. By taking a psychophysical approach and dynamically manipulating signals during female choice I show that the decision-making process is iterative and open-ended. In many species, particularly lek-breeders, females do not encounter males one at a time, as is assumed in models of threshold-based choice (Real 1990); therefore, the utility of distinguishing the validity of sample- and threshold-based decision rules requires reconsideration. The approach I take here places importance on achieving a level of stimulus control over a female during choice to parcel out experimentally the signaling conditions essential to commitment to an initial choice. I show that during mate approach, females continue to gather information about differences between males and use this information to modify their mate choice decision. In general, these results speak to the sensitivity of receivers and the dynamic nature of communication in a way that I believe expands our understanding of decision-making generally and mate choice specifically.

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## **Chapter 4: Female Túngara Frogs Vary in Commitment to Mate Choice**

### **ABSTRACT**

Mate choice studies most often examine female preferences based on population responses, thus potentially overlooking individual differences in behavior. Moreover, such studies typically use invariant stimulus conditions to infer preferences. By using population responses and static stimulus presentations, it is difficult to thoroughly understand the complexity of the mate choice process, including variation present between-individuals. Here, I investigated phonotactic mate choice behavior in female túngara frogs (*Physalaemus pustulosus*) in response to temporally dynamic presentations of male advertisement calls. I tested females on repeated trials to examine individual differences and found considerable variation in the extent to which females update their mate choice decisions. Females in my study were bimodally distributed and thus broadly classified as either committed or uncommitted to an initial mate approach. I compared body condition measures of females differing behaviorally and determined that females with larger residual body masses were more committed to initial mate choices than less massive individuals, despite the fact that all females were in reproductive condition and field-collected in amplexus. My results suggest that anuran phonotaxis, once considered to be a highly stereotyped behavior, is more complex and variable than previously thought.

### **INTRODUCTION**

Communication in nature is dynamic and acoustic signaling in particular usually occurs over brief timescales. Most studies of auditory behavior, however, use repetitive trains of identical stimuli to simplify the experimental conditions under which receiver

behavior is observed. Such designs, while useful, limit the examination of the temporal dynamics involved in signal-receiver interactions. This is especially true in mate choice studies wherein acoustic signals act as a key trait used by receivers in nature to select mates, and especially in lek-breeding species because multiple signalers often compete simultaneously for female attention (Gerhardt and Huber 2002, Ryan 2009).

Furthermore, most mate choice studies examine preferences using population assays, rather than individuals. In some cases, this has perpetuated a typological thinking about behavior that promotes the idea that there is “a female response.” This limits a thorough understanding of individual variation in sexual behavior, and thus sexual selection. In studies that have explored individual variation in sexual behavior, the focus has typically been on examining differences in male traits and their relationship to mating success (reviewed in Andersson 1994). Such studies often focus on trade-offs between the costs of maintaining elaborate traits and the benefits such traits engender through increased attractiveness to females (Møller and de Lope 1995). A much smaller fraction of research has explored the role of individual differences in female choice, even though such patterns might be expected given individual variation in attributes such as age, experience, and body condition among females (Höglund and Alatalo 1995). Condition-dependent expression of preferences, though not addressed in most studies, might optimize female investment in the mate choice process (Jennions and Petrie 1997).

Female preferences might vary in two fundamental ways. First, there may be variation in “preference functions,” the order in which females rank prospective mates; second, females may vary in “choosiness,” the effort an individual is willing to invest in mate assessment (after Jennions and Petrie 1997). In this way, the costs associated with choosiness can affect mating patterns without appreciable variation in preference functions. If these costs vary by individual, then patterns of mate choice may vary while

preference functions remain fixed. It is important to consider such sources of variation as they can affect, among other things, the rate and direction of evolution by sexual selection and provide a window into the mechanistic basis of mate choice (Jennions and Petrie 1997). The proximate causes of variation in female preferences might arise through genetic differences (i.e., polymorphisms), environmental conditions (e.g., predation risk), or differences in reproductive condition, such as the urgency with which females must mate.

A few studies have examined polymorphisms in female preferences. Morris et al. (2003) found substantial variation among females in their preferences for vertical bar patterns in male swordtail fish, *Xiphophorus cortezi*. There was a correlation between the presence of visual patterns in females and the strength of their preference for the same trait in males, suggesting that trait and preference are correlated through a genetic mechanism. In satin bowerbirds (*Ptilonorhynchus violaceus*) there are individual differences in preference due to female age (Coleman et al. 2004); older females attended to male display intensity while their younger counterparts selected males based on their bower ornamentation. Similarly, Moore and Moore (2001) showed that female cockroaches (*Nauphoeta cinerea*) become less choosy as they age. In addition to age effects, female preferences may differ due to their reproductive condition. In midwife toads, for example, female preferences were most consistent during the mated stage. Consistency of phonotactic preferences for the 1.8 kHz call over the 1.5 kHz call were lower at pre- and post-mating time points compared to the mated time point (Lea et al. 2000). In standard phonotaxis tests, female túngara frogs (*Physalaemus pustulosus*) prefer complex to simple calls and this preference is shared by all females (i.e., there are no repeatable individual differences; Kime et al. 1998, Ryan et al. 2003). Further,

willingness to accept a less attractive male call reaches a peak in mated female túngara frogs compared to pre- and post-mated time points. (Lynch et al. 2005a).

In amphibians, females might differ in a subtler manner within the mated time point. As female anurans mature their clutch they face a diminishing window of time with which to find a suitable mate, in some cases oviposition eventually occurs in the absence of a male (Pope 1931, Coe 1974), including in túngara frogs (pers observ). Consistent with this is the prediction that females facing greater urgency to mate will be less choosy (i.e., less willing to invest time in protracted mate assessment or sampling) without necessarily any change in their preference function. In other words, more urgent females might still evince a preference for a more attractive mate over a less attractive alternative if the costs of selecting either mate are equal—if, however, the costs associated with the more attractive mate are high, only those females that do not urgently need to mate will persistently express such a preference.

In a previous study I found that females of a neotropical frog (*Physalaemus pustulosus*) are sensitive to changes in time and space of preferred male call types. Females reversed their phonotactic approach in approximately 50% of trials in which the preferred and non-preferred calls were switched between two opposing speakers (see Chapter 3; Baugh and Ryan in review (b)). In the remaining 50% of trials, females continued on the trajectory of their initial approach despite stimulus switching, thus maintaining their commitment to the same calling male even though his call became less attractive. Here, I explore individual differences in this behavior by repeatedly testing females.

Only a few studies have pursued dynamic mate choice in any animals, including anurans. Dyson et al. (1994) found that female reed frogs (*Hyperolius marmoratus*) will reverse direction after initially approaching (by one body length) a preferred stimulus (the

leading call) if the stimuli are switched. Similarly, Gerhardt et al. (1996) examined the preference for pulse number in *Hyla versicolor* and demonstrated that females, after initially approaching a high pulse number call, would reverse directions if this stimulus were suddenly switched with the less preferred call. These studies provide evidence that at least in a few species of frogs, decision-making involves temporal updating. The extent to which this type of behavior varies individually has, to my knowledge, not been addressed except in the present study. I believe there is much to learn about female variation by closely examining not only the preferences evinced by females, but also the process of mate choice itself. One central question is how exactly are mating decisions made? This question can be answered by manipulating a female's options during the decision-making process.

### **The System**

Túngara frogs are small frogs (ca. 30 mm snout-vent length, SVL) distributed throughout much of Central America (Weigt et al. 2005). During the breeding season (May through December) males vocally advertise to females using a species-typical call, known as the “whine” or simple call (Ryan 1985). Males can produce a complex advertisement call by ornamenting the whine with one to seven suffixes known as “chucks” producing what are known as “whine-chuck” calls. Females use advertisement calls to localize and select a male amongst a chorus; by making physical contact with a male, females select a mate, after which the male mounts and clasps the female in a posture known as amplexus. In laboratory two-choice phonotaxis tests, the whine-chuck calls are strongly preferred to whine calls (85% preference strength; Ryan 1985, Ryan and Rand 2003a). In addition, female túngara frogs exhibit strong preferences for calls of higher amplitude over lower amplitude, presumably resulting in attraction towards nearer

males and thus reducing the travel time and distance required to reach the chosen male (Rand et al. 1997).

## **METHODS**

### **Animals**

I conducted experiments during the breeding season between the months of June and September in 2007 and 2008 at facilities for the Smithsonian Tropical Research Institute in Gamboa, Panamá (9°07.0'N, 79°41.9'W). I collected frogs as amplexant mated pairs from breeding aggregations between 1900 and 2200 h and performed behavioral testing between 2000 and 0400 h; all females were tested and measured before oviposition. Animals were held in small dry plastic bags in dark, quiet conditions before testing. I marked individuals with a unique toe-clip combination to prevent re-sampling, following the Guidelines for the Use of Live Amphibians and Reptiles in Field Research, compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR). After behavioral testing, I measured body mass using a digital balance (to the nearest 0.1 g) and SVL using digital calipers (to the nearest 0.01 mm) before returning females to their original site of collection within 12 h. In total, I tested 105 females in 338 trials; the final data set that resulted included 60 responsive females and 260 successful mate choices (i.e., 76.9% of trials resulted in choices).

### **Stimuli and experimental design**

I used two synthetic stimuli (whine, whine-chuck), which share the identical whine component (see “W” and “Wc” in Figure 20). I synthesized these stimuli based on the average values for the parameters of the calls in the study population by shaping sine waves using custom software (J. Schwartz, Pace University; sample rate 20 kHz and 8

bit). I calculated mean values for the population based on the calls from 50 males recorded in July 1996 with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003b). All playbacks were done using a two-choice design (Figure 22). Stimuli were matched for the peak amplitude of the whine before playback and broadcast from small ADS L210 speakers located in the center of the two poles of the arena. The chuck of the complex call was twice the peak amplitude of the whine. The whines in these signals consist of only the fundamental frequency; it has been shown previously that the upper harmonics of the whine do not significantly contribute to mate choice in the laboratory (Ryan and Rand 1990, Rand et al. 1992).

Frogs were tested under infrared light in a sound-attenuating chamber (Acoustic Systems, Austin, Tx) measuring 2.7 X 1.8 X 1.78 m. Before each subject was tested, I calibrated both speakers to 82 dB SPL (re. 20  $\mu$ Pa) at the center of the arena (1.35 m from each speaker) using the whine stimulus and a GenRad 1982 SPL meter. At the beginning of each trial each subject was placed under a cone at the center of the acoustic chamber (i.e., release point; Figure 22) for three minutes while the two stimuli were broadcast antiphonally at a rate of one call per two seconds from each speaker. The cone was then raised remotely and the phonotactic behavior was monitored via a ceiling-mounted infrared camera connected to a television monitor located outside the chamber.

The chamber was divided symmetrically by boundaries (hereafter “switch boundaries”) at a distance of 75 cm from the poles of the chamber (ca. 20 body lengths from release point; for example configuration see Figure 22). In a previous study using static presentations of whine versus whine-chuck stimuli, I showed that upon crossing the boundary nearest the whine-chuck speaker, responsive female túngara frogs continued

their approach trajectory selecting the whine-chuck speaker (i.e., were “committed”, see Chapter 3) in 100% of trials (Baugh and Ryan in review (b)). These dashed lines were visible only to the human observer (outlined by transparencies on the monitor). In the present study, one of the two speakers initially broadcast the whine-chuck stimulus; this “target” speaker was selected randomly and then alternated between tests and females to minimize any potential side bias (see Results) while the opposite speaker broadcast the whine. When the subject crossed the switch boundary nearest the preferred stimulus (whine-chuck), the human observer pressed a key on the playback computer’s keyboard that initiated a custom program in the acoustic software program Signal®; these programs introduced a 500 ms delay to prevent the premature occlusion of a stimulus, and then each program performed a custom operation suited to the question addressed in the given experiment.

I scored a phonotactic choice when a frog approached one of the speakers within a 10 cm radius without simply following the perimeter of the wall. A frog failed to exhibit a phonotactic choice if it was motionless for the initial 5 min after the cone was raised or during any 2-min interval thereafter, or if the animal failed to make a choice within 15 min after the cone was raised. In approximately 85% of trials females approach the whine-chuck stimulus, a necessary prerequisite for these experiments. In the remaining instances, in which females failed to approach the whine-chuck and instead approached and selected the less preferred stimulus (whine), these responses were recorded but not analyzed in the present study. Because all female túngara frogs exhibit a preference for the whine-chuck (Kime et al. 1998), it is unlikely that omitting these uncommon trials influences my sample.

### ***Experiment 1: Individual differences in reversal frequency***

In Experiment 1 I addressed individual differences in the likelihood of commitment to an initial approach. Here, the two stimuli were switched and the amplitude of the distant stimulus (the one that was not initially approached) was amplified by an amount that equilibrated the mean peak amplitude along the switch boundary (2 dB; this was determined empirically; see Chapter 3; Baugh and Ryan in review (b)).

Three behaviors were scored and a fourth one was calculated: (1) choice: (whether the subject made a choice and if so, whether it involved a reversal or a choice for the initially approached speaker (a non-reversal)). (2) latency to the switch boundary (time lapsed between lifting the cone and crossing the switch boundary nearest the target speaker), (3) overall latency to choice (total time lapsed between lifting the cone and the choice), and (4) the latency to choice following stimulus switching (overall latency minus switch boundary latency).

I tested each female in multiple trials back-to-back until six trials were successfully completed (requiring a mean of 6.44 trials/female) and measured the number of reversal choices that each female performed across these six trials (minimum = 0, maximum = 6;  $N = 40$  females)). To explore the shape of this reversal distribution I determined its kurtosis and compared that to a sampling distribution of kurtosis values drawn from a Monte Carlo simulation (R statistical package) of 10,000 replicates. Kurtosis provides a measure of the strength of a single peak in a sample distribution (small kurtosis values indicate a weak single peak). As an additional method of estimating individual differences I calculated an intra-class correlation (ICC; i.e., repeatability). Repeatability studies typically estimate ICC by calculating the ratio of between-subject variance ( $V_b$ ) to total variance (between + within-subject; Cummings

and Mollaghan 2006, Bell et al. 2009). This formula, however, requires continuous data. Binary data can be analyzed for ICC combining a method developed by Larsen and Merlo (2005) and Marti (pers. comm.; University of Texas Statistics Consulting Services), in which ICC is calculated as:  $(V_b) / (\pi^2 / (3 + V_b))$ . I used this formula to estimate the ICC for the binary (reverse/non-reverse) data on choice. I estimated  $V_b$  using a multilevel logistic regression analysis (SAS, Inc.) following Larsen and Merlo (2005).

From a linear regression of body mass versus SVL for subjects in Experiment 1 I calculated residual body mass for each female (SVL and body mass were not measured for 6 females in this experiment). Also, a body condition index was calculated by obtaining the residual values from a linear regression of the cubed root body mass on SVL and dividing those values by the SVL, following the approach used by Baker (1992), Howard et al. (1997), Howard and Young (1998), and Leary et al. (2008). I used linear regression to evaluate further the degree of reversibility and its correlation with body condition.

I determined if female condition predicted commitment. I classified females dichotomously as either reversible (more than 3 reversal choices; i.e., “uncommitted”) or non-reversible (less than 3 reversal choices; i.e., “committed”) and compared the SVL, body mass, residual body mass, and body condition index for these groups using a repeated measures MANOVA (SPSS 16.0). To ensure that repeatedly testing females did not influence behavior in any systematic way across trials I performed a repeated measures MANOVA (SPSS 16.0) for the three latency measures and a non-parametric Cochran’s Q test for the dichotomous reversal/non-reversal choice data. I examined the time costs of reversal behavior with paired t-tests (two-tailed) on each of the three latency measurements between trials involving a reversal to trials involving a non-reversal within females.

### ***Experiment 2: Individual differences amongst reversible females***

In this experiment I addressed a different source of variation in female commitment— variation in the extent of reversibility amongst reversible (uncommitted) females. The identical stimulus switching operation carried out in Experiment 1 was conducted in Experiment 2 with one exception; here, I repeatedly switched the stimuli each time a female crossed a switch boundary nearest the whine-chuck during the course of a single trial (i.e., “looped switching”) until a choice was eventually made. Provided a female reversed following the initial stimulus switching, I repeatedly switched stimuli after every switch boundary crossing until the female eventually chose the whine without switching. I scored two behaviors ( $N = 20$  females): (1) total number of reversals performed before a choice was made (2) overall latency to choice. I used a linear regression to examine the continuous relationship of latency to choice and the number of reversals.

### ***Experiment 3: Oviposition in responsive and unresponsive females***

I also determined if reproductive state influenced whether females make a mate choice. I estimated reproductive condition simply as whether or not the females mated and constructed a nest with a male at the cessation of testing. In 2008 I tested 144 females in conspecific phonotaxis trials. Of these females, 132 responded by making a choice in at least one trial and the remaining 12 females never responded. Following testing, all females were reunited with the male that they were collected in amplexus with, and each pair was placed in a small plastic container with 1 cm of water and held in a dark, room temperature (26 C) cooler for 12 hours. After this holding period I determined which pairs oviposited (túngara frogs produce a “foam nest” on the water surface; Ryan 1985).

## RESULTS

I found no evidence of a side bias (left:right choices: 122:113; two-tailed exact binomial  $P = 0.60$ ; Baugh and Ryan in review (b)).

### Experiment 1: Individual differences in reversal frequency

#### *Choices*

The population reversal frequency in Experiment 1 was 47.9% (i.e., 52.1% non-reversal). This was similar to the reversal frequency found in a separate study in which females were tested in the same condition but only in two back-to-back trials rather than six (44.3% reversal frequency; see Chapter 3; Baugh and Ryan in review (b)). From the distribution of these responses (Figure 23) it appears that this approximately 50% reversal frequency was due to an underlying bimodal distribution, in which approximately half the females tend to be non-reversible while the other half tend to be reversible. The observed kurtosis of this distribution was quite small (1.55), placing it in the tail end of the Monte Carlo sampling distribution of kurtosis values ( $P = 0.06$ ). Out of the six opportunities to choose for each female, they most commonly reversed either only once or five times (Figure 23). The estimation of between-subject variance from the multilevel logistic regression analysis was quite high (1.0275), generating an ICC (repeatability) value of 0.237 (ICC values are free to vary between -1 and +1, with more positive values indicating a higher degree of repeatability). In other words, there was more variation between subjects than within-subjects, with 23.7% of the variation between subjects explained by individual differences. This is a modestly high repeatability value for studies of mate preferences, which typically have very low values of repeatability compared to other behaviors (reviewed in Bell et al. 2009).

To ensure that there was no effect of repeated testing on reversals I performed the non-parametric repeated measures Cochran's Q test and found that the probability of reversal was not influenced by trial number ( $Q_5 = 5.043$ ,  $P = 0.411$ ; Figure 24d).

The linear regression for cubic body mass by SVL was significant (Pearson's  $R(32) = 0.617$ ,  $P = 1.0 \times 10^{-4}$  (two-tailed)). I dichotomized females as either reversible ( $> 3$  reversals;  $N = 16$ ) or non-reversible ( $< 3$  reversals;  $N = 15$ ) and compared body condition, body mass, residual body mass and SVL for these two groups using a repeated measures MANOVA. Reversible and non-reversible females differed significantly in body condition index ( $F_{1,29} = 7.703$ ,  $P = 0.010$ , Figure 25a), body mass ( $F_{1,29} = 6.267$ ,  $P = 0.018$ ) and residual body mass ( $F_{1,29} = 7.976$ ,  $P = 0.008$ ), but not in SVL ( $F_{1,29} = 0.380$ ,  $P = 0.543$ ), suggesting that behavioral differences were not simply due to differences in age (túngara frogs, like many ectothermic vertebrates, continue to grow after reaching sexual maturity). Subsequent to identifying this relationship between body condition and behavior, I re-examined data from an independent study of dynamic mate choice (see Chapter 3; Baugh and Ryan in review (b)) to confirm this effect; here again, the linear regression for cubic body mass by SVL was significant (Pearson's  $R(141) = 0.456$ ,  $P < 1 \times 10^{-6}$  (two-tailed)). In this previous study females were tested on two back-to-back dynamic choice trials of whine versus whine-chuck stimuli in which reversal frequencies observed were similar to that seen in the present study. Thus, I dichotomized these females as non-reversible (zero reversals;  $N = 90$ ) and reversible (two reversals;  $N = 53$ ). From this larger dataset I similarly found that reversible and non-reversible females differed significantly and in the same direction in body condition index ( $F_{1,141} = 4.177$ ,  $P = 0.043$ ; Figure 25b), body mass ( $F_{1,141} = 3.938$ ,  $P = 0.049$ ), and residual body mass ( $F_{1,141} = 3.943$ ,  $P = 0.049$ ). The two groups did not differ in SVL ( $F_{1,141} = 0.213$ ,  $P = 0.645$ ).

Finally, I performed linear regressions for the dataset of 6 repeated trials to examine further if females making a greater number of reversals had smaller mass measurements. I found negative correlations in all three measures with a significant effect for the number of reversals performed and body mass (Spearman's  $\rho$  (32) = -0.345,  $P$  = 0.046 (two-tailed)), and non-significant correlations for residual body mass (Spearman's  $\rho$  (32) = -0.327,  $P$  = 0.059 (two-tailed))), and body condition (Spearman's  $\rho$  (32) = -0.313,  $P$  = 0.072 (two-tailed))) (Table 5).

### ***Latencies***

Two of the three latency measurements differed between reversal and non-reversal trials (Table 6). Latency to choice after stimulus switching was significantly greater for trials involving a reversal choice compared to a non-reversal ( $P = 1 \times 10^{-6}$ ). Likewise, latency to overall choice was greater for reversal choices compared to non-reversals ( $P = 6 \times 10^{-6}$ ). Latency to the switch boundary, however, did not differ between these two response categories ( $P = 0.54$ ).

To ensure that repeated testing did not influence latency responses, I examined my three measures of latency across the six repeated trials using repeated measures MANOVA. Mauchly's test of sphericity was violated and therefore I used the Greenhouse-Geisser correction (adjusted degrees of freedom) to generate  $P$ -values. I found that the main effect of trial number on latency was not significant ( $F_{11,22} = 1.116$ ,  $P = 0.395$ ) and that none of the three individual outcomes were significant (boundary latency:  $F = 1.974$ ,  $P = 0.121$ ; latency to choice after switching:  $F = 1.133$ ,  $P = 0.344$ ; and overall choice latency:  $F = 1.979$ ,  $P = 0.105$ ). This suggests that repeatedly testing females does not affect their latency behavior (Figure 24a–c).

## **Experiment 2: Individual differences amongst reversible females**

In this experiment females were required to reverse following the first initial stimulus switching; therefore, the minimum number of reversals was one. Of the twenty females in this study, 10 females reversed only a single time before making a choice, 3 females reversed 2 times, 1 female reversed 3 times, 1 female reversed 4 times, 3 females reversed 5 times, 1 female reversed 9 times, and 1 female reversed 16 times (mean = 3.15, median = 1.5, mode = 1.0, range = 1–16).

I also measured overall latency to choice in this experiment and found a large range (mean  $\pm$  SEM (s):  $250.6 \pm 44.87$ ; range: 70–773). Not surprisingly, females generally had longer latencies given a greater number of reversals (Spearman's  $\rho$  (18) = 0.602,  $P = 0.005$  (two-tailed)).

## **Experiment 3: Oviposition in responsive and unresponsive females**

Across all females, 90.2% produced a foam nest when reunited with the mate they had chosen in the field. The majority of females were responsive, performing conspecific phonotaxis in at least one trial (132 compared to 12 non-responsive frogs). Responsive females were significantly more likely to produce a foam nest (94.6% produced foam nests) compared to non-responsive females (58.3% produced foam nests; Fisher's exact test, two-tailed:  $P = 0.002$ ) despite the fact that all females were collected at breeding sites in amplexus with a male.

## **DISCUSSION**

Because the vast majority of acoustic mate choice studies use static stimulus presentation designs and population assays, important elements of behavior potentially go unnoticed. Here, I demonstrate that female frogs update their decision-making in real time as advertisement signals change and report, for the first time, that the process of

executing a mate choice can vary strikingly between individuals; in my study, females were bimodally distributed in their commitment to an initial mate approach, and these two behavioral classes of females differed in body condition, wherein less committed females had lower body mass compared to more committed females.

A previous study using a static mate choice design demonstrated that female túngara frogs do not exhibit individual differences in preferences for complex over simple calls (Kime et al. 1998). Despite this lack of individual variation in preferences, my study shows that choosiness varies between individuals; here, choosiness is reflected in the likelihood that females reverse their approach in order to select a more attractive signal, despite the fact that such behavior incurs a time cost, and presumably energetic and conspicuousness costs as well (Rand et al. 1997). I also show that this choosiness is linked to variation in body condition. There are a few possible causes of body mass variation, including differences due to clutch size (i.e., number of ova), clutch mass or both. In many anurans clutch size correlates positively with SVL (reviewed in Duellman and Trueb 1986), including tropical anurans that make foam nests, where larger species (SVL) produce more eggs (Crump 1974). Because the two classes of females in my study did not exhibit differences in SVL it is unlikely that body condition differences were due to variation in clutch size, although this possibility cannot be ruled out. Additionally, whereas clutch size in túngara frogs varies considerably, there is no evidence that it does so in a bimodal manner (Ryan 1985, Baugh unpublished data). A more likely cause of body mass variation is differences in clutch mass, which has been shown in other anurans to be tied to clutch hydration, as evaporative water loss and intake can amount to as much as 20% of body mass (Sinsch 1983); thus, the body condition of a female may be strongly influenced by exactly how far she has progressed towards ovulation. In some species of tropical frogs, females lay multiple egg masses in a night of breeding and hydrate eggs

during inter-mating intervals by partially immersing themselves in water (Deullman and Trueb 1986). Female túngara frogs, however, do not mate multiply within a breeding cycle but are often found prior to mating in the chorus ponds, and also spend time in these pools while in amplexus before oviposition. It is unknown just how long females spend hydrating a clutch, but given that non-responsive females were significantly less likely to oviposit (Experiment 3), it is conceivable that there is appreciable variation amongst amplexant females in the urgency with which females must mate. Importantly, mate choice appears to be entirely under female control in this species—there is no evidence of scramble competition mating in túngara frogs (Ryan 1985). Therefore, it is unlikely that amplexant females had been clasped by males without having selected them. Further, while there has been some debate about the assumptions of body condition measures that are based on residuals from length/mass correlations (Green 2001), these measures have been used widely, and in a recent meta-analysis appear to generate useful information (Schulte-Hostedde et al. 2005). Perhaps most importantly, these condition measures are related to behavior in the present study.

My study showed that despite the lack of a polyphenism in preferences for complex calls (Kime et al. 1998), individual differences can arise due to variation in choosiness. This contrasts with findings in other species of anurans. For example, studies of female preferences in midwife toads (*Alytes muletensis*) by Lea (2000) and cricket frogs (*Acris crepitans*) by Ryan et al. (1992) both found size-dependent preferences and attributed these differences to tuning properties of the inner ear that are known to change with body size. Similarly, using the African painted reed frog (*Hyperolius marmoratus*), Jennions et al. (1994) found size-dependent variation between females in their responses to calls differing in frequency, wherein large females appeared to be more sensitive to small differences in frequency and were more consistent in their preference for low

frequency calls than small females. In my study, body size did not differ between reversible (uncommitted) and non-reversible (committed) females, and again all females in my study approached the whine-chuck initially. It is unlikely that differences in frog behavior were due to age differences, as SVL in anurans often correlates positively with age, and no differences in SVL were observed (Halliday and Verrel 1988, Castellano et al. 1999). Instead, my data show that the temporal process of mate choice is correlated with differences in body condition amongst highly responsive and reproductively competent females (Experiment 3 showed that 94.6% of field-collected amplexant females that responded positively in at least one phonotaxis trial oviposited within 12 hours). I suggest that females closer to the time at which they must oviposit are more committed; this is congruent with a previous study that suggested that as female túngara frogs approach the terminal part of their reproductive cycle they are willing to accept less attractive mates (Lynch et al. 2005a).

Because in nature vocal signaling in nature by male túngara frogs is a dynamic process, the results of my study have implications for sexual selection. To maximize survival and reproductive success, males calling behavior should evolve an optimal compromise between the costs of complex call production (acoustic predators prefer complex calls; Ryan et al. 1982) and its benefits (female preference). In addition, I now show that a considerable subpopulation of females is attending to the timing and consistency of complex call production, thus adding another behavior that should be subject to selection. This challenges a popular conception of mate choice by “simple” vertebrates such as frogs, since multiple variables clearly influence the final choice outcome.

Future studies will explore the causes and consequences of behavioral variation in commitment. To better grasp mating urgency, it will be useful to derive a continuous

measure of the latency to oviposit by females differing behaviorally. I predict that more committed females will have shorter latencies to oviposit compared to their more choosy counterparts. As for mechanisms, it is not known how changes in clutch hydration might be linked to the neural differences underlying the behavioral variation. Assuming that hydration state is linked to proximity to oviposition, previous studies have demonstrated that gonadal hormone levels change during the pre- to post-breeding time points and that exposure to calls concomitant with gonadal hormone manipulation increases immediate-early gene expression in the auditory midbrain (Lynch and Wilczynski 2005, Lynch and Wilczynski 2008). Non-steroid mechanisms might also give rise to behavioral variation of the sort observed in this study. Arginine vasotocin (AVT), for instance, has been shown to affect behavioral receptivity in female leopard frogs (*Rana pipiens*) through the accumulation of body fluids that cause a mechanical distention of the abdomen (Diakow 1978), even in the absence of ovarian tissue (Diakow et al. 1978). Female túngara frogs respond more quickly following subdermal injections of AVT (Baugh and Ryan in preparation (a)), but the effects of AVT have not been examined in the context of temporal updating. On a shorter time scale, catecholamines are known to play a role in attentional processes and can interact with gonadal steroid hormones (Sellix et al. 2004). Therefore, administering agonists (e.g., L-DOPA) and antagonists (e.g., MK-801) of dopamine, for example, could prove informative. The consequences of variation in choosiness are presently unknown, but presumably are linked to energetic, time, and conspicuousness costs of increased mate sampling and potentially mate quality. These aspects deserve future research.

This is the first study to my knowledge that explores how body condition is related to updating and decision-making in mate choice behavior. The methods used in the present study enable the inspection of individual differences that would be

unavailable in conventional mate choice experiments. My results suggest that phonotaxis is not merely a two-step procedure of evaluating signals and executing a decision, but rather a flexible and open-ended process. Females vary in the extent to which they continue to gather information about differences between males and use this information to modify their final mating decision. Indeed, in a previous study 25% of females are so committed to an initial approach that discontinuing call broadcast does not interfere with their approach and choice (see Chapter 3; Baugh and Ryan in review (b)). These results speak to the complexity and variability of anuran behavior as well as the dynamic nature of communication in a way that expands our understanding of decision-making generally, and mate choice specifically. It is also important to note that as biologists we tend to recoil at large variances around some mean trait; large variances are especially common for behavioral traits. Individual differences within a species, however, are the raw material for selection and this is particularly true for sexual selection, as differential reproductive success among individuals can be high in frogs (Kluge 1981), including in túngara frogs (Ryan 1985).

In conclusion, I agree with the position by Jennions and Petrie (1997) that the study of mate choice would benefit from greater attention devoted to examining female behavior from the perspective of the individual, and avoiding the typological thinking which often assumes homogeneity amongst females, and to realize that mate choice in many simple vertebrates involves decision-making that is often attributed to only birds and mammals (Ryan et al. 2007).

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## **Chapter 5: Categorical Perception of a Natural, Multivariate Signal: Mating Call Recognition in Túngara Frogs**

### **ABSTRACT**

Categorical perception is common in humans, but it is not known if animals perceive continuous variation in their own multidimensional social signals categorically. There are two components to categorical perception—labeling and discrimination. In the first, continuously variable stimuli on each side of a category boundary are labeled. In the second, there is strong discrimination between stimuli from opposite sides of the boundary, while stimuli on the same side of the boundary are not discriminated. Here, I show that female túngara frogs respond categorically to complex mating calls that vary simultaneously along multiple dimensions and are within the natural range of signal variation. In response to a transect of synthetic stimuli that varied continuously and systematically in seven dimensions, female túngara frogs label mating calls as either conspecific or not conspecific. For pairs of stimuli that differed by the same magnitude, females discriminate those in different categories but not those in the same category. In addition, latency to respond was significantly shorter when stimuli were in the same versus different categories. Because responses to mating calls are critical in generating species recognition and sexual selection, this finding has implications for both animal perception and the influences of mate choice on the tempo and mode of evolution.

### **INTRODUCTION**

Animals use only a fraction of available sensory information to assess their worlds, and different animals can have access to different sets of data emanating from the same environmental or social stimuli (von Uexküll 1909/1985). An animal's perception of the world also can be influenced by how that information is processed. One important

factor is whether stimuli are perceived continuously or in discrete categories. Categorical perception in humans is well known in color, speech, and facial discrimination (Harnad 1987, Diehl et al. 2004, Webster et al. 2004). Other animals exhibit categorical perception of human phonemes (Kuhl 1981, Kluender et al. 1987). In addition, a few non-human animals show categorization of their own signals, including the following: song variants in songbirds (Nelson and Marler 1989), ultrasonic vocalizations in mouse pups (Ehret and Haack 1981), and mating/bat echolocation calls in crickets (Wytenback et al. 1996). In these animal studies, and most human studies, however, stimuli varied along a single dimension. It is not known, therefore, if categorical perception extends to the vast majority of communication signals, which covary over multiple dimensions.

Perception of social signals is a critical stage preceding many social decisions. One of the most important decisions an animal makes is deciding with whom to mate. These decisions are usually informed by signals that vary between and within species (Ryan et al. 2007). In most instances, females choose to mate with conspecifics rather than heterospecifics, and choose the more attractive males among conspecifics (Kirkpatrick and Ryan 1991, Andersson 1994). Mating preferences can lead to reproductive isolation between groups, and this in turn promotes their divergence into different species. In addition, preferences for certain variants of signal traits among conspecifics can generate sexual selection and lead to the evolution of extreme male traits. Thus the perceptual mechanisms underlying mate choice can play an important role in evolution, but they are often ignored (Ryan et al. 2007).

Many studies investigate how signal variation influences mate choice. One result of such studies is the “preference function,” in which the strength of preference is represented as a function of continuous variation in the mating signal (Ritchie 1996, Wagner 1998, Kirkpatrick et al. 2006). The preference function illustrates the nature of

selection on male traits and may influence how traits evolve. Contrary to the results of the present study, all studies to date show that a continuous change in a signal parameter covaries with a continuous change in the strength of preference for that signal. Thus females respond to mating signals, whether comparing the same or different species, as being continuously more-or-less attractive. An alternative perceptual mode that might underlie mate choice is categorical perception, in which stimuli are categorized dichotomously as preferred or non-preferred. There is no evidence to date for categorical perception of mating signals, although few studies have explicitly tested this hypothesis. I do so here by examining female preferences for mating call variation in the túngara frog, *Physalaemus pustulosus*.

Most male frogs produce acoustic signals to attract females for mating, and females discriminate call variation between and within species (Gerhardt and Huber 2002). Females exhibit these preferences through phonotaxis, approach toward a mating call (Ryan 1980, Ryan 1985, Ryan et al. 1990, Ryan and Rand 1995). Although these experiments do not reveal the absolute potential for discriminating among stimuli, i.e., just noticeable differences, they emphasize the more ecologically relevant category of just meaningful differences and are thus more pertinent to the behavioral and evolutionary consequences of perceptual processes (Nelson and Marler 1989, Wytenback et al. 1996, Nelson and Marler 1990).

The túngara frog's mating call is a downward frequency sweep, or "whine", of approximately 300 ms (Figure 26). Males can add "chucks" to the end of this call, which increases its attractiveness. The whine, however, is both necessary and sufficient to elicit phonotaxis from females, and all closely related species have whine-like calls (Ryan and Rand 1995). I examined female preferences in response to a series of synthetic calls that varied continuously between the conspecific whine and the call of an allopatric

heterospecific, *Physalaemus coloradum*. A previous study demonstrated that female túngara frogs recognize the *P. coloradum* call but significantly prefer the conspecific call when given a two-choice test (Ryan and Rand 1995). Seven call parameters were adjusted by the same percentage to synthesize calls intermediate between each of the species' calls (PC series; Figure 26); for example, the call PC25 has a fall time of 298 ms, which is 25% different from the *P. pustulosus* fall time (343 ms) and 75% different from the *P. coloradum* fall time (161.7 ms; Table 7). Thus synthetic calls of any “acoustic distance” (the overall summed difference of all seven call parameters) from the conspecific call can be synthesized, and the difference between any pair of stimuli easily ascertained (Ryan et al. 2003); e.g., PC6 is 6% different from both the conspecific call (PC00) and the PC12 call. I used the following nine calls: PC50, PC37, PC31, PC25, PC18, PC12, PC6, PC00, and PC-6. Synthetic advertisement calls have been used previously to explore mate choice in túngara frogs and among other findings these studies have demonstrated that the synthetic versions of the calls are treated no differently from natural stimuli (Rand et al. 1992).

Reproductively active females were collected in Gamboa, Panamá, and tested with two-choice phonotaxis experiments to assess their preferences. I used two-choice rather than one-choice tests because they better approximate natural mate choice conditions. In addition, two-choice tests are more sensitive to differences between stimuli and are thus conservative when testing for categorical perception (Phelps et al. 2006).

## **METHODS**

Female frogs were collected between 1900 and 2200 hours and tested between 2200 and 0600 hours during the summers of 2006 and 2007. Following testing, females were toe-clipped to avoid re-testing them, and returned to their original capture site within 12 h of collection. Collection permits in Panamá were approved by the Autoridad

Nacional del Ambiente and animal care and use approval by the University of Texas IACUC #6041701. I tested 167 females (410 choice trials) during the course of this study and for the repeated measures component I randomized the order in which the experiments were conducted. The average number of tests that each female participated in was 2.57 and the average number of transects per female was 1.11. Females were highly responsive with successful choices performed in 91.1% of trials.

Frogs were tested under infrared light in a sound attenuating chamber (Acoustic Systems, Austin, TX) measuring 2.7 x 1.8 x 1.78 m. Female behavior was observed on a video monitor connected to an infrared camera on the chamber ceiling. At the beginning of each phonotaxis test a female was placed under a small cone in the center of the chamber floor. I broadcast the two test stimuli antiphonally from speakers located in the center of the walls at the two poles of the chamber. Stimuli were presented at a rate of one call per two seconds from each speaker and speakers were calibrated to a peak amplitude of 82 dB SPL (re 20  $\mu$ Pa). Between successive trials, stimuli were alternated between the two speakers to avoid a stimulus x side bias. I concurrently tested for side bias using identical stimuli and those results as well as an analysis of the present data strongly demonstrate the lack of a side bias in this study.

Stimuli were presented for three minutes while the female was under the cone. Then the cone was raised and a phonotactic choice was scored if a female approached one of the two stimuli within a 10 cm radius without simply following the wall. A female failed to exhibit a phonotactic choice if she was motionless for the initial 5 min after the cone was raised or during any 2 min interval thereafter. Finally, a female did not exhibit phonotaxis if she failed to make a choice within 15 min after the cone was raised. In addition to measuring choice I also recorded latency to choice.

I used a binomial test (one-tailed) to evaluate each experiment against the null prediction of no preference ( $P = 0.5$ ); there was the *a priori* expectation that females prefer the conspecific call to alternatives (Ryan et al. 1990). For repeated measures experiments I also used the non-parametric Cochran's  $Q$  test to evaluate each female's response under one experiment compared to the other two experiments. Response latencies for repeated-measures experiments were converted to subject  $z$  scores and analyzed with a one-way ANOVA.

## RESULTS

In the first battery of tests, I used the average synthetic version of the conspecific call (PC00) as a referent for exploring categorization. Females discriminated between PC00 and the four most different calls (PC12,  $P = 0.001$ ; PC25,  $P = 0.006$ ; PC37,  $P = 0.057$ ; PC50,  $P < 0.001$ ; Figure 27). There was no preference, however, when PC00 was paired with the two most similar calls (PC-6,  $P = 0.412$ ; PC6,  $P = 0.588$ ; Figure 27). A control experiment in which PC00 was paired with itself yielded no preference (PC00,  $P = 0.412$ ; Figure 27). The results of these experiments demonstrated labeling; when compared to conspecific calls, the test calls were considered to be either conspecific or not conspecific.

Another hallmark of categorical perception is a lack of discrimination between stimuli within a category and strong discrimination between stimuli of the similar difference but in different categories. Given the suggestion of category labeling in the above results, I tested the hypothesis that females should discriminate between two stimuli across categories, but not within each category, even as the stimuli within each pair differed by the same amount. I used a repeated-measures design wherein each female completed the following three experiments: PC00 versus PC6; PC6 versus PC12; and, PC12 versus PC18. I predicted that females would discriminate between PC6 versus

PC12 but would not discriminate between the other two pairs even though the calls in each pair differed by 6%. As predicted, there was no preference in the two within-category experiments of PC00 versus PC6 ( $P = 0.443$ ; Figure 28a) and PC12 versus PC18, although the data suggested a trend towards a preference ( $P = 0.059$ ). There was, as predicted, a strong preference in the between-category test, PC6 versus PC12 ( $P = 0.001$ ). In addition, there was a significant overall Cochran's  $Q$  for the three experiments ( $Q = 6.81, P = 0.03$ ), resulting from a difference in preference between PC00 versus PC6 and between PC6 versus PC12 ( $Q = 6.54, P = 0.01$ , Bonferonni corrected). The remaining two pair-wise comparisons were not significantly different (Figure 28a). Further support of categorical perception derives from pairwise tests at the extremes of each category—PC18 versus PC25 ( $P = 0.412$ ), and PC25 versus PC31 ( $P = 0.412$ ).

A common finding in categorical perception studies is that response times differ for within- and between-category discriminations (Pisoni and Tash 1974, Bornstein and Korda 1984, Campanella et al. 2000). Pisoni and Tash (1974) and Bornstein and Korda (1984) found slower response times for evaluating between-category stimuli compared to equivalently different within-category stimuli; this effect, however, was not analyzed statistically. Thus I compared response latencies for the experiments in Figure 28a. The one-way ANOVA generated a marginally significant overall effect of experiment on latency ( $F = 2.95, df = 2, P = 0.055$ ; Figure 28b). Planned independent contrasts showed that the latencies for the between-category comparison were significantly greater than the two within-category comparisons ( $t_{147} = 2.406, P = 0.017$ ; Figure 28b).

## DISCUSSION

These data show that female túngara frogs respond to stimulus variation in a manner that is similar to categorical perception in humans and in some other animals. Unlike previous animal studies, I demonstrate that categorical perception can occur in a

mate choice context and is not restricted to a single acoustic dimension, as has been tested in other studies of categorical perception, but can also emerge in response to acoustic signals that vary in multiple dimensions, a common feature of virtually all social signals. In addition, the synthetic stimuli used in this study comprised a range of acoustic variation that falls within that of the study population's calls as evaluated using multidimensional scaling (Figure 29). This natural intraspecific variation has not yet been examined for patterns of continuous or categorical mate choice.

One interpretation of this study is that mate preference and stimulus variation is not always a simple function as is implicit in studies of sexual selection by female choice. If that is the case, then we might expect male traits to evolve in a more punctuated mode (Gould 2002, Turner 1984). It also might suggest reexamination of the notion that females assess continuous quantitative variation in display traits as indicators of male genetic quality (Zahavi and Zahavi 1997, Kokko et al. 2003). Recent studies have investigated in great detail the scaling of male signals in sexual selection (Kodric-Brown et al. 2006), my study suggests that scaling of female preferences for these signal is worthy of similar attention. Also, because studies of categorical perception of vocal communication signals in songbirds have focused on male behavioral (Nelson and Marler 1989, Prather et al. 2009) and neural responses (Prather et al. 2009), there is an intriguing opportunity to extend these tests to females to further assess the influence of this perceptual mode on female choice.

I suggest that understanding perception of mating signals is critical to a deep understanding of how species recognition evolves and how sexual selection generates some of the most extreme behaviors and morphologies in the animal kingdom.

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## Conclusions

Diversity in behaviour is great in nature, and this fact should be reflected in our studies. The influence and acceptance (even the romance) of ethology lie largely in reopening our eyes to the richness of life, so apparent to nineteenth-century naturalists yet lost with the rise of behaviorism in psychology and mechanism in biology (Burghardt and Bekoff 1978).

This quote expresses the scientific and intellectual potential of the comparative approach—that much can be learned about the living world by exploring the peculiarities between species. It also signals the value of comparing individuals within a species, whether they are animals at different developmental life history stages, or reproductive adults of different sexes. Lastly, this quote captures the notion that it is a worthwhile endeavor for us to re-evaluate our taxonomic biases about the behavioral and cognitive abilities of animals, a position that is emerging undeniably as a theme in contemporary behavioral biology (Van Lawick-Goodall 1971, Burghardt 1978, Mather 1995, Thomas 1996, Hauser et al. 2000, Pepperberg 2000, Cole and Adamo 2005, Baugh et al. 2008).

The research presented in this dissertation focuses on auditory behavior in frogs. There are key advantages, and a few disadvantages, in using frogs to explore questions about perception, and the development and execution of behavior. One of the most important advantages is the robustness of phonotaxis in adults. Along with the abundance of túngara frogs in Panamá, these two factors enabled the use of large-scale experiments, which were necessary given the subtlety of some of the behaviors studied (e.g., categorical perception, Chapter 5). Further, because túngara frogs are a lek-breeding anuran, and hence receivers have evolved to make decisions and compare multiple males under complex acoustic conditions, the experiments in Chapters 3 and 4 were achievable. Females are remarkably sensitive and attendant to the dynamic nature of signals. The results from the temporal updating studies demonstrate unequivocally that mate choice in

this species is an active process; females actively reject certain males in favor of another caller despite a more intense stimulus from the rejected caller, contrasting with the predictions of passive mate choice (*sensu* Parker 1983). Examining the sometime subtle details of decision-making was largely made possible by the use of acoustic signals—the ability to control the precise timing of signal onset and offset, not unlike a natural communication scenario, was an essential prerequisite for these studies. Studies examining other sensory modalities such as olfaction can be acutely constrained in this regard (even with the use of olfactometers). Although the decision-making behavior examined in this work focused on mate choice, it is necessary to bear in mind that this is only one class of reproductive decisions that females make. Because túngara frogs are a lek species, females must also choose a lek—a relatively understudied topic of investigation. This point underscores an important empirical detail—males also perform temporal updating and this very fact illustrates the role of lek selection that the sexes share. In other words, one probable interpretation of temporal updating in male frogs is that it functions simply as a way to guide males to the most attractive lek, toward which females will also be attracted.

Performing developmental studies with anurans presents both benefits and costs. On the one hand, rearing animals in the laboratory is easy and relatively inexpensive; however, there are no postmetamorphic staging criteria for anurans, nor are there external traits or genetic assays that identify a froglet's sex (unlike in birds; Griffiths et al. 1998). Nonetheless, even in immature animals, stimulus-elicited responses can be obtained. Developing methods for animal training such as reinforcement learning would greatly improve the potential of anurans as behavioral models of perception and cognition; unfortunately, attempts to do so have been met with only limited success (see Yerkes 1905, Macphail 1982, Simmons and Moss 1995, Elepfandt 1996). In Chapter 2, I show

that frogs recognize and respond to conspecific mating calls well before sexual maturity, demonstrating that such behavior, assumed previously to operate as a purely sexual behavior exhibited by adults, has a premature origin. This suggests that despite morphological and physiological changes that occur during postmetamorphic development (see Chapter 1), species-typical perception of sound remains intact and normal. Whether this is due to compensation that occurs centrally during development, or simply by virtue of the developmental changes being relatively mild, remains unknown. Thus, in a qualitative sense, the suggestion that juvenile frogs are simply “miniature adults” is perhaps not entirely inappropriate. I demonstrate quantitatively, though, that conspecific phonotaxis is increasingly expressed as froglets grow toward adulthood. This suggests that changes do occur during development and these are principally motivational in nature. Finally, I show that there are differences between the sexes during reproductive adulthood, including fine-scale movements near the sound source, and that gonadotropins have opposing effects on the sexes.

To my knowledge the study presented in Chapter 2 is the first report of auditory behavior in subadult frogs. I discussed the various interpretations of these findings in the context of possible functions of this behavior and describe other systems that exhibit preparatory development in the absence of plasticity (vocal or auditory learning), leading to the suggestion that a more fundamental principle of constraint in developing sensory and motor systems is present and has been under-recognized. This research is of general interest for several reasons: (1) it challenges established notions of what constitutes a sexual behavior in a vertebrate class that has been a model for understanding sexual selection; (2) it raises questions about sex differences and similarities with respect to presumed function; (3) it introduces a system for studying developmental origins of behavior with the advantage of a behavioral task performed by both sexes—this is unlike

systems such as songbirds, in which different tasks performed by the sexes (e.g., copulation solicitation in females and vocalizations in males) introduce confounding variables that hinder interpretation; and (4) it urges investigators to reconsider typological classifications of behavior, such as “hard-wired,” which can have a deadening effect on interest in the study of behavioral development in many organisms. This logic also applies to Schneirla’s concern (see Chapter 1) about assumptions regarding separate mechanisms for each type of behavior. For instance, in some sense we might view phonotaxis as a single type of behavior. As I showed in Chapter 2, however, this behavior is performed by frogs across developmental time and by both sexes, suggesting that there is more than a single behavioral trait present. Phonotaxis does differ between the sexes and not simply in a quantitative sense (i.e., males and females do not exhibit differences in the frequency with which they express conspecific phonotaxis, suggesting that sex differences are not simply due to some gradation in the activation of underlying shared neural pathways); only reproductive females, for example, exhibit the perseverance component to this behavior. Further, because hCG had opposing effects on adult males and females, there might be a complex variety of underlying mechanisms regulating phonotaxis in frogs. Alternatively, because the downstream consequences of hCG differ between the sexes, the source of sex differences could in fact be tightly linked to differences in gonadal steroid systems.

One important caveat is necessary regarding the use of anurans for studies of behavioral development; juvenile phonotaxis is expressed infrequently (approximately 15% of trials result in a conspecific choice) and as such, developmental studies of this behavior are time-intensive. This limitation might be ameliorated to some extent through the use of smaller phonotaxis arenas, thus requiring less locomotor investment by juveniles; such an augmentation, of course, carries the increased risk of false positives

and hence the need for larger sample sizes. Another possibility not explored in these experiments would be the use of population assays to increase sampling efficiency; the drawback here is the potential for interactive effects amongst juveniles within a trial. Worthwhile future developmental studies might include physiological characterization of auditory responses to conspecific signals, comparative behavioral and neural studies in related taxa (e.g., *P. petersi*, the sister species), and field studies aimed at determining what, if any, the function of juvenile phonotaxis might be.

In Chapter 3 I examine an underappreciated component of mate choice studies—the temporal process of decision-making. I decompose mate selection into temporal and spatial components and demonstrate that reproductive decision-making is an open-ended process. A female's final mate choice depends upon consistent signaling information from a preferred male, and becomes increasingly decided as a female nears the approached male. Interfering with a consistent stream of advertisement calls in real time can elicit predictable changes in a female's mate choice. I demonstrate what those predictable changes in mate choice are by manipulating features of the advertisement calls as females make their approach. This was a large-scale study, having over 1000 mate choices conducted across 21 different experiments. Collectively, these experiments demonstrate that certain acoustic features of signals (complexity, amplitude, continued presence, and the intrinsic attractiveness of natural calls) and a non-acoustic feature of the environment (ambient light levels) influence a female's commitment to a mate choice. In general, these results speak to the sensitivity of receivers and the dynamic nature of communication in a way that I believe expands our understanding of decision-making generally and mate choice specifically.

I expand on the findings of Chapter 3 through an investigation of individual differences in dynamic female mate choice in Chapter 4. By again by controlling the

exact timing of stimulus presentation, I examine the extent to which females differ individually in choosiness, as measured by their commitment to an initial approach. I show that females differ strikingly, and bimodally, in their willingness to accept a male call that has suddenly decreased in attractiveness. Further, I demonstrate that this behavioral variation correlates with female body condition; more massive females (i.e., more reproductively advanced) are less choosy. I discuss these results in the context of other studies demonstrating individual differences in female choice, describe parallel explanatory processes underlying such variation, and recommend future investigations to further clarify the mechanisms responsible for this natural variation. Together, Chapters 3 and 4 emphasize the importance of isolating and measuring the *process* of mate choice and present novel methods for studying such a process. The results, particularly from Chapter 4, suggest that what are sometimes typological ways of thinking about female behavior, invoked in phrases such as “the female response” deserve reconsideration.

Chapter 5 takes another route towards revealing the diversity and complexity of behavior in this anuran. This chapter shows that female túngara frogs perceive mating calls categorically, either as conspecific or not conspecific. Although the phenomenon of categorical perception is well established in humans, there are few non-human examples of this perceptual mode, and in these rare instances the signals used to explore perception vary only in a single dimension (Nelson and Marler 1989, Wytenbach et al. 1996, Prather et al. 2009). Here, I demonstrate that social signals varying in multiple dimensions are perceived categorically by reproductively active females. Moreover, the stimuli used in this study fall within the natural range of variation for the study population—this is significant as it suggests that a categorical mode of perception might influence the nature of sexual selection and the evolution of mating displays. This study will be of general interest for the following reasons: (1) it extends what was once thought

of as a uniquely human trait associated with language (see Hauser et al. 2002) to another animal, the first "lower" vertebrate now known to employ categorical perception; (2) it shows for the first time that animals can apply this ability to complex signals that vary in many dimensions simultaneously, not just over a single dimension such as duration (e.g., Nelson and Marler 1989, Prather et al. 2009). Since nearly all communication signals are multi-dimensional it suggests even more generality than previously appreciated; (3) it demonstrates for the first time categorical perception during mate choice and it has important implications for how mate choice can drive speciation and the evolution of extreme male traits through sexual selection; (4) it provides an example of categorical perception of vocal communication signals that are not learned (for a contrasting example see Prather et al. 2009); and finally, (5) it contributes to an emerging paradigm uniting the cognitive and ecological sciences.

Because mate choice constitutes one of the most important decisions animals make, perhaps it comes as no surprise that such decision-making can be complex, and context- and state-dependent. Although the research presented here focuses on receiver behavior, the results spur questions about specific adaptations in signalers. For example, given the highly sensitive responses of reproductive females to interruptions in male calling behavior, including simple changes in call complexity, we might anticipate that males minimize abrupt transitions from complex to simple; Bernal et al. (in press (b)) provide some support for this prediction by showing that males adjust the complexity of vocalizations gradually, and rarely transition abruptly from multi-chuck calls to whine-only calls. Expanding on this issue through field studies or naturalistic laboratory studies might yield additional insights into the dynamic interactions that occur between signalers and between signaler and receiver. As more studies explore communication from the perspective of the decision-making process, our understanding of communication, and

hence our capacity to predict and interpret aspects of this important class of animal behavior, is enhanced. This dissertation reminds us to bear in mind that subtle communication behavior and capabilities in organisms might be present yet occur infrequently, only under certain contexts, only at a short distance from a signaler, or may vary significantly within- and between individuals.

**Table 1.** Descriptive statistics for ontogeny of phonotaxis (Chapter 2, Experiment 2) (page 1 of 2).

Stimulus 1   Stimulus 2	Time point 1	Time point 2	Time point 3	Time point 4
<b>Total Number of Choices</b>				
Silence   Silence	0   1	0   1	1   2	2   2
Noise   Silence	1   1	1   1	1   1	0   5
PE-0.37   Silence	0   0	2   0	3   4	4   1
Whine   Silence	2   1	2   1	7   0	3   2
Whine-chuck   Silence	4   0	2   1	8   0	7   1
Whine   PE-0.37	1   1	4   0	11   1	7   3
Whine-chuck   Whine	3   0	2   1	4   0	7   5
<b>Mean Total Path Length (<math>\pm</math> 95% CI, cm)</b>				
Silence   Silence	163.50 (47.43)	237.49 (52.18)	243.85 (54.58)	226.15 (52.26)
Noise   Silence	209.43 (51.21)	160.54 (30.49)	233.86 (45.22)	324.96 (75.94)
PE-0.37   Silence	120.08 (36.73)	211.13 (43.40)	242.72 (75.94)	266.97 (68.84)
Whine   Silence	145.25 (39.84)	139.09 (25.81)	146.79 (47.17)	179.66 (55.18)
Whine-chuck   Silence	132.60 (52.55)	173.57 (40.81)	175.44 (41.83)	167.08 (42.31)
Whine   PE-0.37	101.53 (31.02)	172.10 (29.32)	194.32 (53.51)	215.27 (44.28)
Whine-chuck   Whine	156.30 (54.01)	116.68 (26.05)	142.04 (34.93)	178.00 (39.00)
<b>Mean Perseverance Path Length After Choice (<math>\pm</math> 95% CI, cm)</b>				
Silence   Silence	n/a (n/a)   23.60 (n/a)	n/a (n/a)   27.53 (n/a)	31.46 (n/a)   13.77 (3.85)	13.77 (3.85)   33.43 (26.98)
Noise   Silence	15.73 (n/a)   62.93 (n/a)	7.87 (n/a)   23.60 (n/a)	7.87 (n/a)   31.46 (n/a)	n/a (n/a)   27.53 (16.35)
PE-0.37   Silence	n/a (n/a)   n/a (n/a)	31.96 (0.98)   n/a (n/a)	20.98 (18.53)   23.60 (13.16)	51.13 (30.99)   3.22 (n/a)
Whine   Silence	17.70 (11.56)   7.87 (n/a)	19.75 (7.54)   35.40 (n/a)	16.39 (6.42)   n/a (n/a)	38.02 (34.57)   31.46 (15.42)
Whine-chuck   Silence	34.41 (18.98)   n/a (n/a)	9.83 (3.85)   23.60 (n/a)	21.14 (8.73)   n/a (n/a)	21.63 (10.29)   23.60 (n/a)
Whine   PE-0.37	31.464 (n/a)   11.80 (n/a)	25.56 (15.58)   n/a (n/a)	17.69 (4.12)   7.87 (n/a)	17.04 (15.11)   37.36 (3.85)
Whine-chuck   Whine	31.46 (15.42)   n/a (n/a)	19.67 (30.83)   35.40 (n/a)	10.82 (8.55)   n/a (n/a)	10.11 (6.48)   17.70 (11.59)
<b>Mean Latency to Choice After Choice (<math>\pm</math> 95% CI, s)</b>				
Silence   Silence	n/a (n/a)   270 (n/a)	n/a (n/a)   540 (n/a)	490 (n/a)   581.50 (238.14)	540.50 (398.85)   583.50 (71.54)
Noise   Silence	645 (n/a)   521 (n/a)	290 (n/a)   380 (n/a)	482 (n/a)   195 (n/a)	n/a (n/a)   450.20 (157.98)
PE-0.37   Silence	n/a (n/a)   n/a (n/a)	398 (192.08)   n/a (n/a)	673.67 (38.47)   429.50 (204.87)	396 (127.48)   483 (n/a)
Whine   Silence	646 (129.35)   600 (n/a)	737.5 (4.90)   470 (n/a)	499.57 (75.10)   n/a (n/a)	333 (200.90)   590 (352.79)
Whine-chuck   Silence	550 (178.35)   n/a (n/a)	728 (94.08)   480 (n/a)	532.75 (168.49)   n/a (n/a)	403 (97.79)   680 (n/a)
Whine   PE-0.37	365 (n/a)   620 (n/a)	597.25 (127.35)   n/a (n/a)	535.27 (112.39)   204 (n/a)	542.86 (106.04)   267.67 (371.71)
Whine-chuck   Whine	501 (307.26)   n/a (n/a)	487.50 (73.50)   280 (n/a)	553.75 (140.73)   n/a (n/a)	343 (199.43)   449.60 (235.51)
<b>Mean Weighted Association Time Score (<math>\pm</math> 95% CI)</b>				
Silence   Silence	7.35 (13.45)   7.65 (9.76)	17.70 (16.26)   37.20 (24.51)	22.96 (20.57)   39.94 (27.49)	41.05 (26.94)   27.24 (20.79)
Noise   Silence	8.99 (7.82)   10.38 (10.26)	19.75 (29.17)   31.83 (26.74)	29.87 (29.69)   64.34 (43.98)	14.25 (16.26)   72.02 (32.85)
PE-0.37   Silence	22.37 (18.08)   9.11 (9.63)	31.31 (27.96)   5.70 (6.23)	48.59 (27.23)   31.79 (27.83)	44.59 (37.74)   42.59 (27.69)
Whine   Silence	11.89 (12.60)   9.40 (12.53)	24.76 (27.69)   12.02 (19.28)	97.51 (51.49)   1.38 (2.70)	65.13 (44.70)   21.60 (22.40)
Whine-chuck   Silence	29.55 (28.70)   9.34 (12.93)	37.40 (27.41)   13.76 (16.72)	79.03 (46.11)   8.12 (7.65)	122.54 (64.26)   13.52 (18.46)
Whine   PE-0.37	6.82 (8.93)   8.84 (9.96)	44.49 (32.89)   2.55 (2.82)	90.77 (48.39)   8.95 (10.10)	101.29 (49.65)   42.04 (28.02)
Whine-chuck   Whine	12.95 (10.98)   10.26 (10.83)	25.36 (25.20)   18.40 (27.50)	59.80 (41.86)   17.53 (20.49)	124.07 (68.40)   58.79 (46.89)

**Table 2.** Descriptive statistics for ontogeny of phonotaxis (Chapter 2, Experiment 2) (page 2 of 2).

Stimulus 1   Stimulus 2	Time point 5	Time point 6	Field-caught Adults
<b>Total Number of Choices</b>			
Silence   Silence	4   0	1   1	1   1
Noise   Silence	2   5	1   5	1   1
PE-0.37   Silence	6   5	5   1	2   0
Whine   Silence	11   1	15   0	18   0
Whine-chuck   Silence	10   1	15   1	15   0
Whine   PE-0.37	16   5	14   1	11   1
Whine-chuck   Whine	8   7	11   4	13   3
<b>Mean Total Path Length (<math>\pm</math> 95% CI, cm)</b>			
Silence   Silence	199.65 (86.47)	135.78 (54.96)	234.01 (106.00)
Noise   Silence	197.21 (59.05)	219.22 (75.41)	211.89 (117.58)
PE-0.37   Silence	241.97 (80.97)	282.05 (71.81)	164.69 (82.64)
Whine   Silence	123.89 (28.33)	180.82 (58.66)	455.90 (184.26)
Whine-chuck   Silence	181.39 (62.02)	213.79 (61.29)	435.25 (156.37)
Whine   PE-0.37	283.08 (103.99)	178.76 (68.11)	377.57 (144.65)
Whine-chuck   Whine	251.43 (80.86)	239.54 (73.81)	369.37 (154.93)
<b>Mean Perseverance Path Length After Choice (<math>\pm</math> 95% CI, cm)</b>			
Silence   Silence	34.09 (13.60)   n/a (n/a)	35.40 (n/a)   27.53 (n/a)	23.60 (n/a)   23.60 (n/a)
Noise   Silence	21.63 (19.27)   41.95 (31.57)	1.00 (n/a)   28.32 (35.36)	23.60 (n/a)   23.60 (n/a)
PE-0.37   Silence	33.82 (17.68)   21.63 (20.27)	130.58 (163.87)   31.46 (n/a)	37.36 (26.98)   n/a (n/a)
Whine   Silence	28.71 (13.55)   11.79 (n/a)	170.17 (100.07)   n/a (n/a)	306.77 (178.22)   n/a (n/a)
Whine-chuck   Silence	25.56 (8.77)   11.80 (n/a)	160.41 (81.24)   19.67 (n/a)	390.15 (193.02)   n/a (n/a)
Whine   PE-0.37	32.69 (12.89)   32.78 (12.85)	121.08 (104.47)   31.46 (n/a)	293.15 (163.55)   23.59 (n/a)
Whine-chuck   Whine	35.40 (28.51)   20.97 (14.02)	112.27 (71.89)   17.70 (19.53)	238.92 (187.26)   277.93 (471.55)
<b>Mean Latency to Choice After Choice (<math>\pm</math> 95% CI, s)</b>			
Silence   Silence	543 (225.59)   n/a (n/a)	390 (n/a)   20 (n/a)	240 (n/a)   650 (n/a)
Noise   Silence	575 (107.80)   382.20 (75.00)	135 (n/a)   351.80 (182.88)	375 (n/a)   63 (n/a)
PE-0.37   Silence	268.17 (144.80)   353.60 (192.37)	346.50 (187.11)   370 (n/a)	272.5 (347.89)   n/a (n/a)
Whine   Silence	289.91 (117.14)   200 (n/a)	274.08 (85.96)   n/a (n/a)	226.11 (74.22)   n/a (n/a)
Whine-chuck   Silence	367.80 (139.41)   760 (n/a)	348.27 (118.44)   65 (n/a)	145.86 (37.20)   n/a (n/a)
Whine   PE-0.37	380.63 (105.42)   363 (227.09)	384.79 (107.97)   73 (n/a)	264.63 (104.14)   273 (n/a)
Whine-chuck   Whine	422.75 (118.87)   235.71 (104.84)	377.64 (139.75)   452 (214.00)	300 (119.86)   415.66 (232.88)
<b>Mean Weighted Association Time Score (<math>\pm</math> 95% CI)</b>			
Silence   Silence	48.22 (28.93)   37.31 (30.05)	37.28 (28.13)   13.44 (18.16)	45.07 (41.51)   91.04 (60.52)
Noise   Silence	33.36 (23.79)   101.75 (49.96)	23.75 (17.41)   47.57 (26.57)	28.14 (21.63)   49.76 (38.89)
PE-0.37   Silence	59.01 (36.20)   74.31 (48.55)	70.63 (40.77)   46.01 (24.51)	35.17 (29.29)   15.54 (14.38)
Whine   Silence	199.55 (77.11)   24.13 (26.97)	233.35 (81.31)   4.20 (7.79)	400.88 (106.44)   0.00 (0.00)
Whine-chuck   Silence	153.52 (67.76)   27.53 (20.11)	207.40 (72.08)   13.66 (22.11)	422.44 (113.66)   4.19 (6.27)
Whine   PE-0.37	173.32 (67.02)   33.89 (21.96)	178.14 (69.26)   13.22 (16.97)	231.04 (106.17)   14.62 (18.11)
Whine-chuck   Whine	101.88 (48.56)   116.64 (58.32)	155.91 (67.98)   69.12 (49.81)	260.36 (112.45)   55.23 (56.25)

**Table 3.** Reversal results for dynamic mate choice experiments (Chapter 3). Stimuli: W = synthetic whine, Wc = Synthetic Whine-chuck, W (n1) = d1 “Oc” natural whine (from Ryan and Rand 2003b), Wc (n1) = d1 natural whine-chuck, W (n2) = d3 “M” natural whine (ibid), Wc (n2) = d3 natural whine-chuck, W (n3) = d8 “Sd” natural whine (ibid), Wc (n3) = d8 natural whine-chuck. Convention for naming experiments: first position: sex (M = male, F = female); second position: distance (cm) from face of target speaker at which stimuli are switch or pseudo-switched; third position: stimuli manipulation (S = switched, P = pseudoswitched, C = ceased); fourth position: decibel level at which distant stimulus is boosted by upon stimulus manipulation; fifth character: unique identifier for experiment (a-u).

Experiment	Initial Stimuli Ch 1—Ch 2	Final Stimuli Ch 1—Ch 2	Amplitude Boost (dB)	No. Body Lengths from RP to Switch Boundary	Reversal Choices	Non-Reversal Choices	No Response	Total Choices	N	% Reversals
F-75-S-0-a	W—Wc	Wc—W	0	20	24	56	41	80	40	30.0
F-75-S-2-b	W—Wc	Wc—W	2	20	62	78	26	140	70	44.3
F-75-P-2-c	W—Wc	W—Wc	2	20	0	40	18	40	20	0
F-60-S-6-d	W—Wc	Wc—W	6	25	17	23	7	40	20	42.5
F-100-S-1.3-e	W—Wc	Wc—W	1.33	10	26	14	3	40	20	65.0
F-75-S-4-f	W—Wc	Wc—W	4	20	18	22	15	40	20	45.0
F-75-P-2-g	W—Wc	W—W	2	20	5	35	10	40	20	12.5
F-75-S-2-h	Wc—Wc	Wc—W	2	20	15	25	0	40	20	37.5
F-75-P-2-i	Wc—Wc	Wc—Wc	2	20	4	36	4	40	20	10.0
F-75-S-2-j	W—W	Wc—W	2	20	21	19	3	40	20	52.5
F-75-P-2-k	W—W	W—W	2	20	3	37	10	40	20	7.5
F-75-S-2-l	W(n1)—Wc(n3)	Wc(n1)—W(n3)	2	20	18	42	14	60	30	30.0
F-75-S-2-m	W(n3)—Wc(n1)	Wc(n3)—W(n1)	2	20	23	37	30	60	30	38.3
F-75-S-2-n	W(n1)—Wc(n2)	Wc(n1)—W(n2)	2	20	17	43	10	60	30	28.3
F-75-S-2-o	W(n2)—Wc(n1)	Wc(n2)—W(n1)	2	20	38	24	33	62	31	63.3
F-75-S-2-p	W—W3c	W3c—W	2	20	18	42	19	60	30	30.0
F-75-S-2-q	W—W3c	W3c—W	2	20	11	29	14	40	40	27.5
F-75-C-2-r	W—Wc	W—silence	2	20	40	0	10	40	20	100
F-75-C-0-s	W—Wc	silence—silence	n/a	20	0	5	15	20	20	0
M-75-S-2-t	W—Wc	Wc—W	2	20	14	26	65	40	20	35.0
F-75-S-2-u	W—Wc	Wc—W	2	20	8	32	6	40	20	20.0

**Table 4.** Mean latencies ( $\pm$  SEM) pooled from experiments *b*, *l*, *m*, *n*, and *o* for reversal and non-reversal choices along with paired t-tests for each.

Latency measure	Non-reversal mean latency (s) $\pm$ SEM	Reversal mean latency (s) $\pm$ SEM	Paired t-test
<b>Boundary</b>	82.7 $\pm$ 11.5	78.0 $\pm$ 9.6	$t = 0.46, df = 63, P = 0.649$
<b>Choice after switch</b>	34.9 $\pm$ 3.3	95.4 $\pm$ 9.5	$t = 6.75, df = 64, P < 1 \times 10^{-6}$
<b>Overall choice</b>	132.6 $\pm$ 14.4	177.5 $\pm$ 14.7	$t = 4.51, df = 78, P = 0.00002$

**Table 5.** The number of reversal choices (out of 6 trials) was negatively correlated with female body mass (Spearman's rho (32) = -0.345, P = 0.046 (two-tailed)), but not for residual body mass (Spearman's rho (32) = -0.327, P = 0.059 (two-tailed)) or body condition index (Spearman's rho (32) = -0.313, P = 0.072 (two-tailed)).

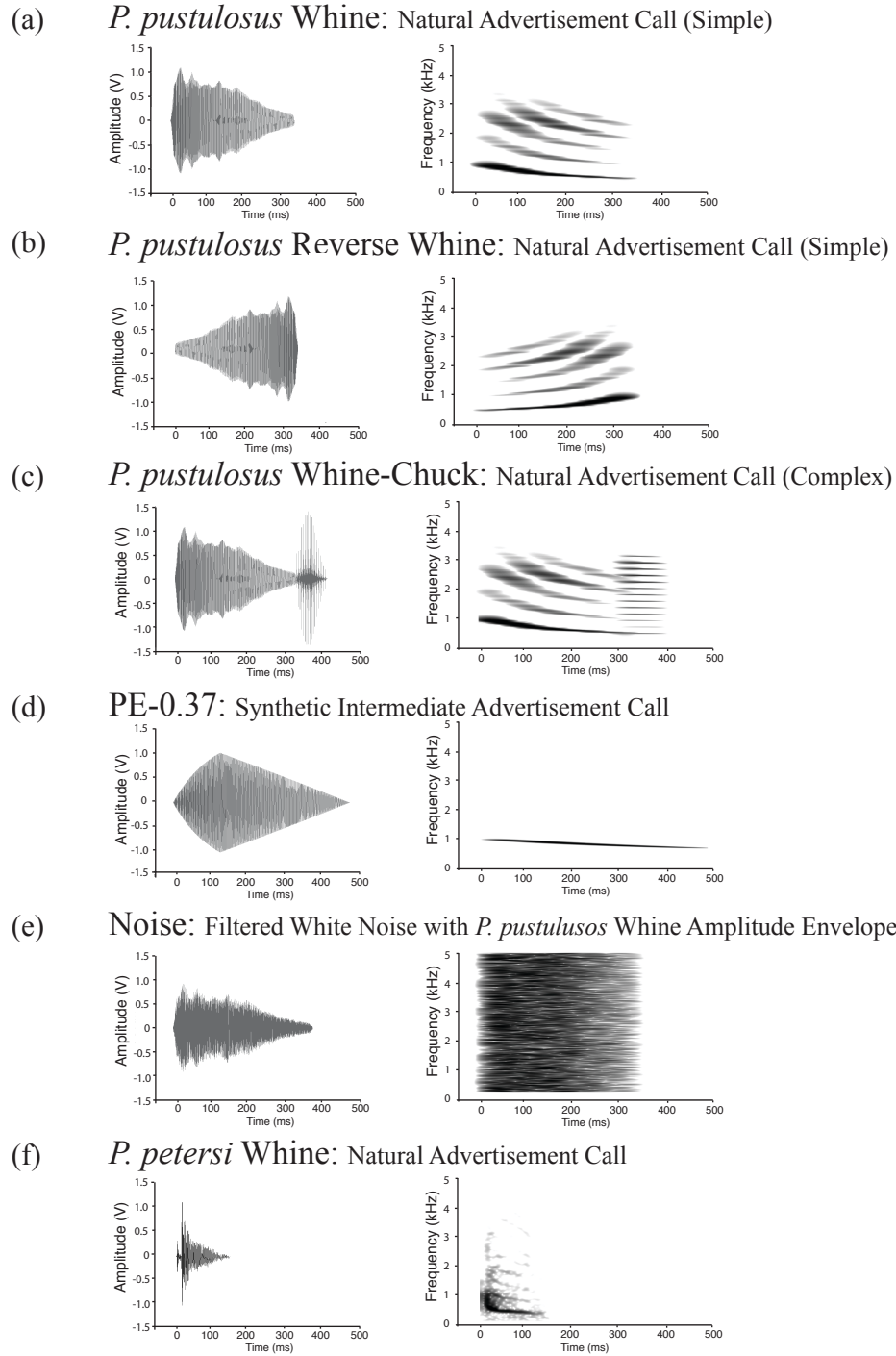
Subject ID	No. Reversals	SVL (mm)	Body Mass (g)	Residual Body Mass	Body Condition Index
1314	0	27.84	2.47	0.39408	0.002878
0002	0	29.89	2.18	-0.14147	-0.000883
1023	0	27.68	2.08	0.02817	0.000358
2012	1	27.12	2.25	0.26598	0.002135
1201	1	30.06	2.46	0.12124	0.000771
1311	1	27.12	1.71	-0.27601	-0.002095
1312	1	27.19	2.24	0.24163	0.001951
2035	1	31.70	2.40	-0.12940	-0.000881
0005	1	31.10	2.56	0.09417	0.000472
1032	1	27.94	2.25	0.17015	0.001353
1240	1	29.00	2.28	0.07070	0.000569
0004	2	30.01	3.04	0.70420	0.004084
1123	2	28.69	2.26	0.08768	0.000711
1450	2	23.64	1.65	0.07514	0.000638
1154	2	25.82	1.74	-0.09392	-0.000699
1155	3	25.33	1.74	-0.03446	-0.000220
1445	3	27.63	2.41	0.36314	0.002718
1153	3	26.75	2.49	0.54712	0.004170
2023	4	30.21	2.01	-0.34065	-0.002222
1121	4	25.06	1.19	-0.55025	-0.005631
2033	4	29.05	1.66	-0.55226	-0.003988
2034	4	28.85	1.63	-0.56740	-0.004165
1000	4	25.38	2.05	0.26956	0.002389
1223	4	24.90	1.63	-0.08917	-0.000793
1125	5	27.62	1.95	-0.09966	-0.000606
1452	5	29.08	2.58	0.36115	0.002400
2011	5	30.04	2.26	-0.07737	-0.000469
0001	5	27.81	1.95	-0.12333	-0.000780
0003	5	29.93	2.43	0.10375	0.000680
1015	5	30.69	2.22	-0.19491	-0.001242
1020	5	26.84	1.70	-0.25361	-0.001951
1115	5	28.04	1.94	-0.16077	-0.001055
1225	5	25.66	1.93	0.11416	0.001089
1122	6	26.83	1.60	-0.35141	-0.002822

**Table 6.** Mean latencies ( $\pm$  95% CI) to switch boundary, choice after stimulus switching, and overall choice during trials when females reversed and did not reverse (averaged within subjects for reversal and non-reversal trials). Paired t-tests.

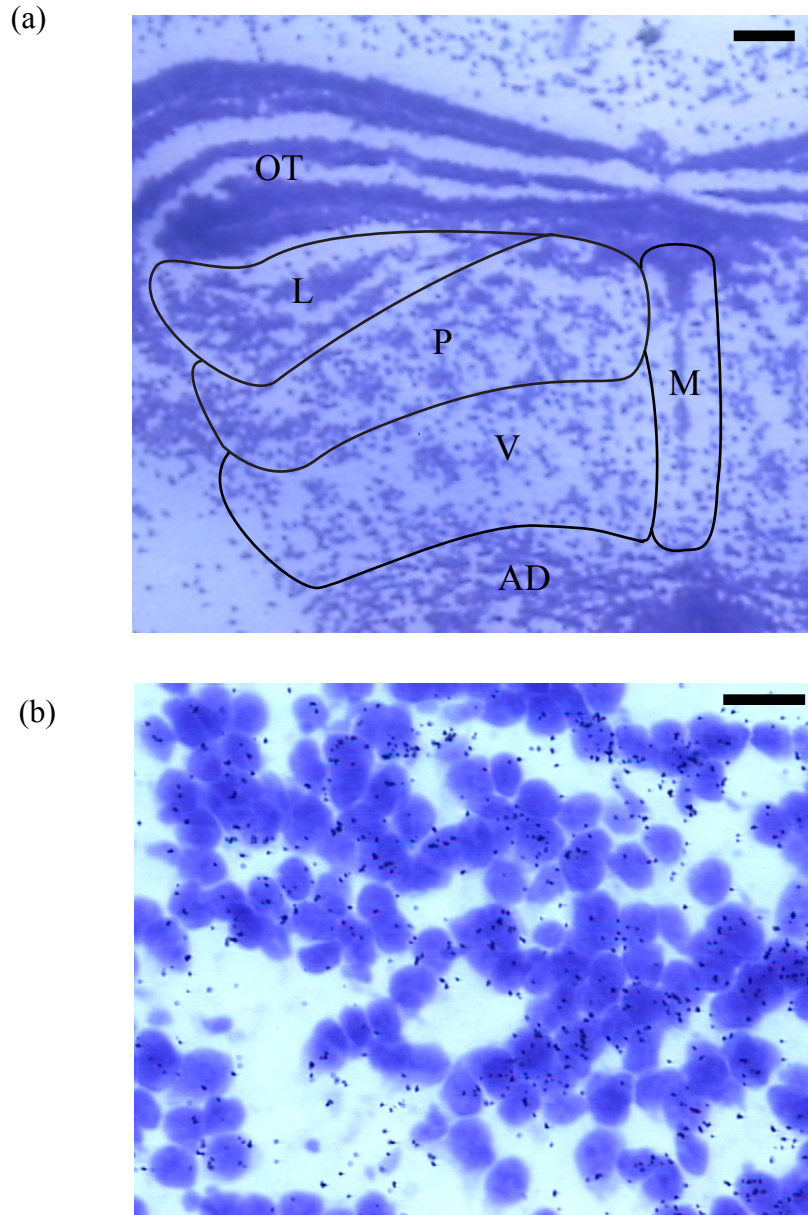
Measurement	Non-reversal	Reversal	Paired t-test
Latency to switch boundary ( $\pm$ SEM, s)	60.3 ( $\pm$ 7.8)	64.7 ( $\pm$ 8.9)	$t(29) = 0.61, P = 0.54$
Latency to choice after switch ( $\pm$ SEM, s)	34.0 ( $\pm$ 5.0)	72.9 ( $\pm$ 6.3)	$t(29) = 6.28, P = 1 \times 10^{-6}$
Latency to overall choice ( $\pm$ SEM, s)	94.2 ( $\pm$ 10.7)	137.2 ( $\pm$ 13.2)	$t(29) = 5.50, P = 6 \times 10^{-6}$

**Table 7.** The mean values of fundamental frequency parameters for nine stimuli used in Chapter 5. Stimulus PC100 was not used in this study but is shown here for comparison.

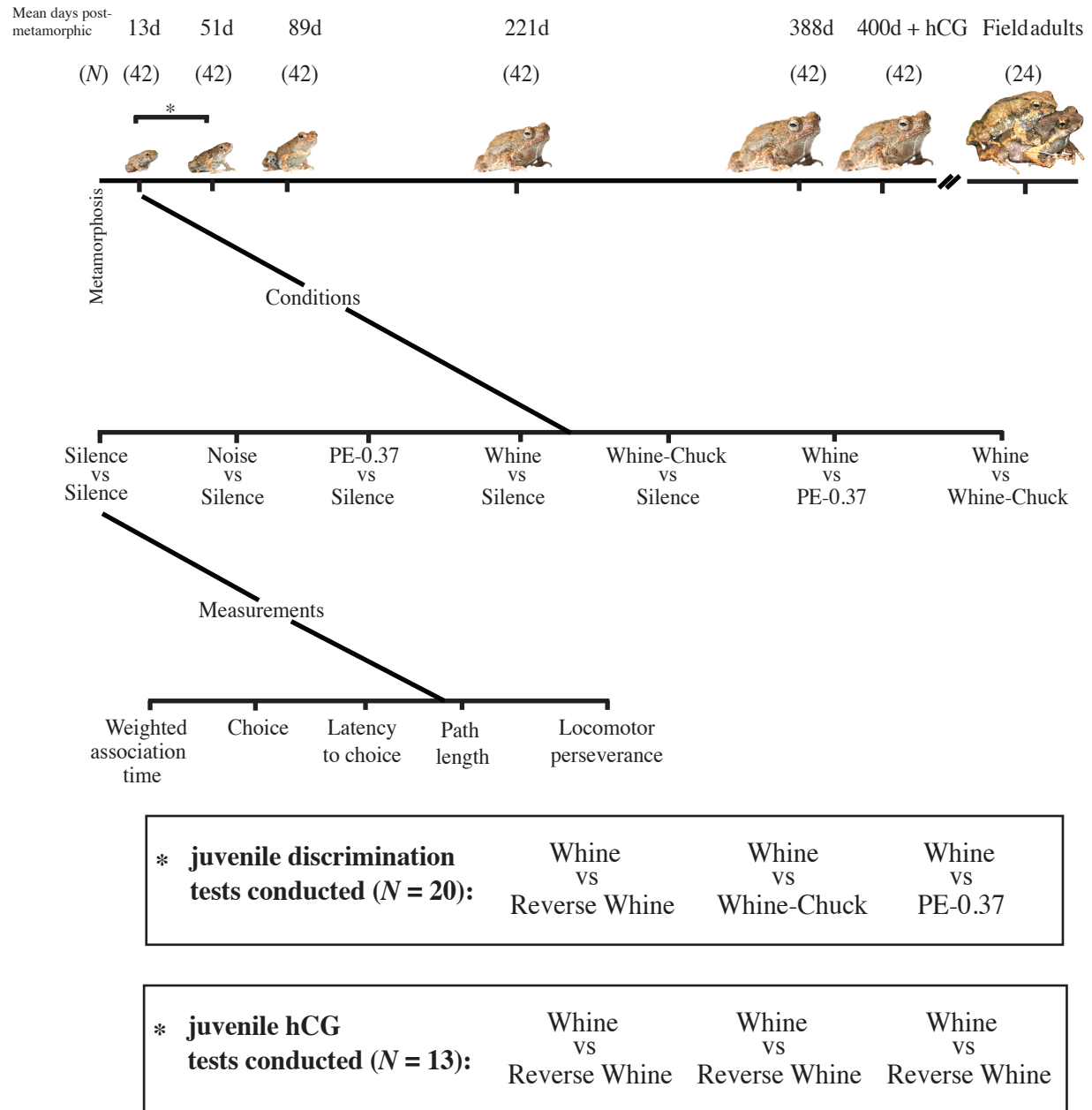
Stimulus	Maximum frequency (Hz)	Final frequency (Hz)	Rise time (ms)	Fall time (ms)	Frequency sweep shape	Fall shape	Rise shape
PC-6	866	475	22.24	353.88	0.34	0.47	0.32
PC00	884	484	24.00	343.00	0.34	0.48	0.33
PC6	893	493	25.76	332.12	0.34	0.49	0.34
PC12	920	501	27.53	321.24	0.35	0.51	0.34
PC18	937	510	29.29	310.37	0.35	0.52	0.35
PC25	958	520	31.35	297.68	0.35	0.54	0.36
PC31	976	529	33.11	286.80	0.35	0.55	0.36
PC37	994	537	34.88	275.92	0.36	0.56	0.37
PC50	1032	556	38.70	252.35	0.36	0.59	0.38
PC100	1180	628	53.40	161.70	0.39	0.71	0.44



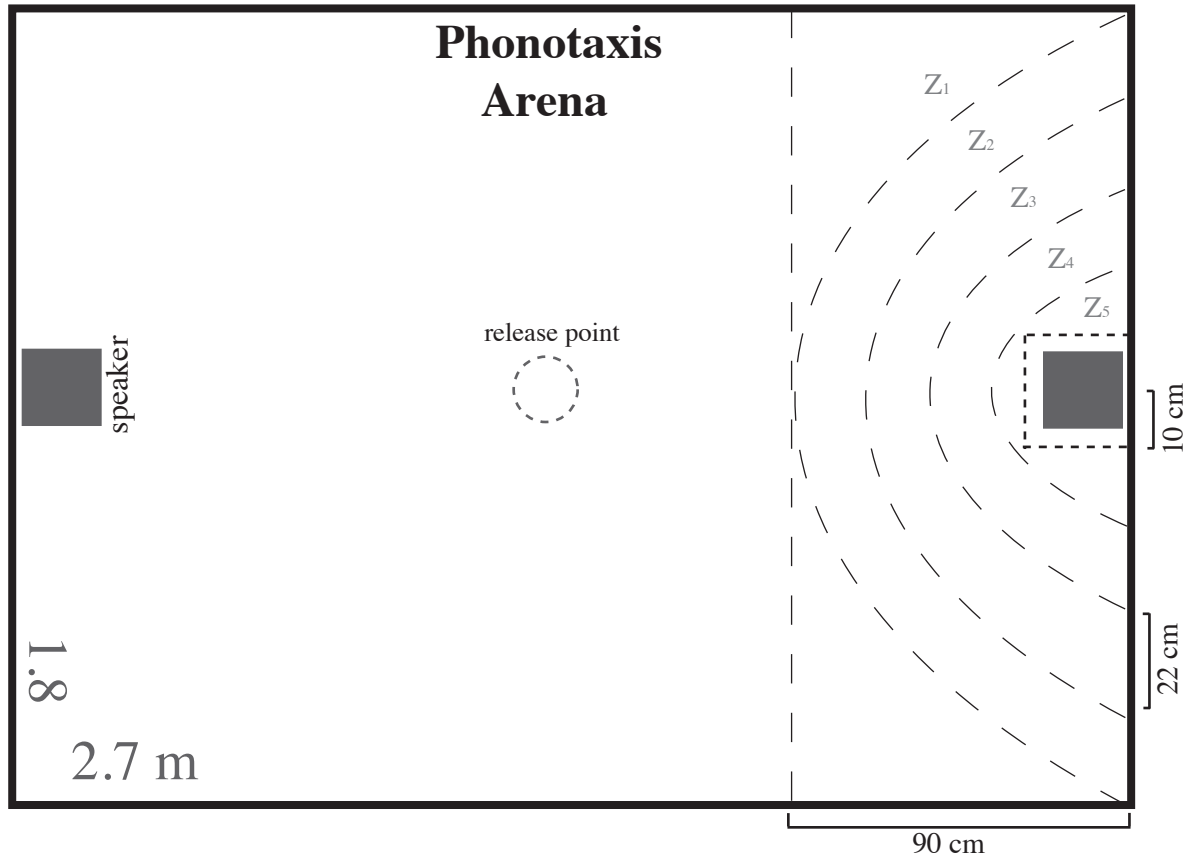
**Figure 1.** Oscillograms and spectrograms of stimuli used in developmental studies. (a) natural whine advertisement call (stimulus “M” from Ryan and Rand 2003b); (b) temporally reversed version of (a); (c) natural whine-chuck advertisement call, identical to (a) with a single chuck; (d) synthetic call that is intermediate between *P. pustulosus* and *P. eneseophae* (ibid); (e) white noise bandpass filtered (100–5000 Hz) and shaped with the amplitude envelope of (a); (f) natural whine advertisement call of *P. petersi* recorded in Ecuador.



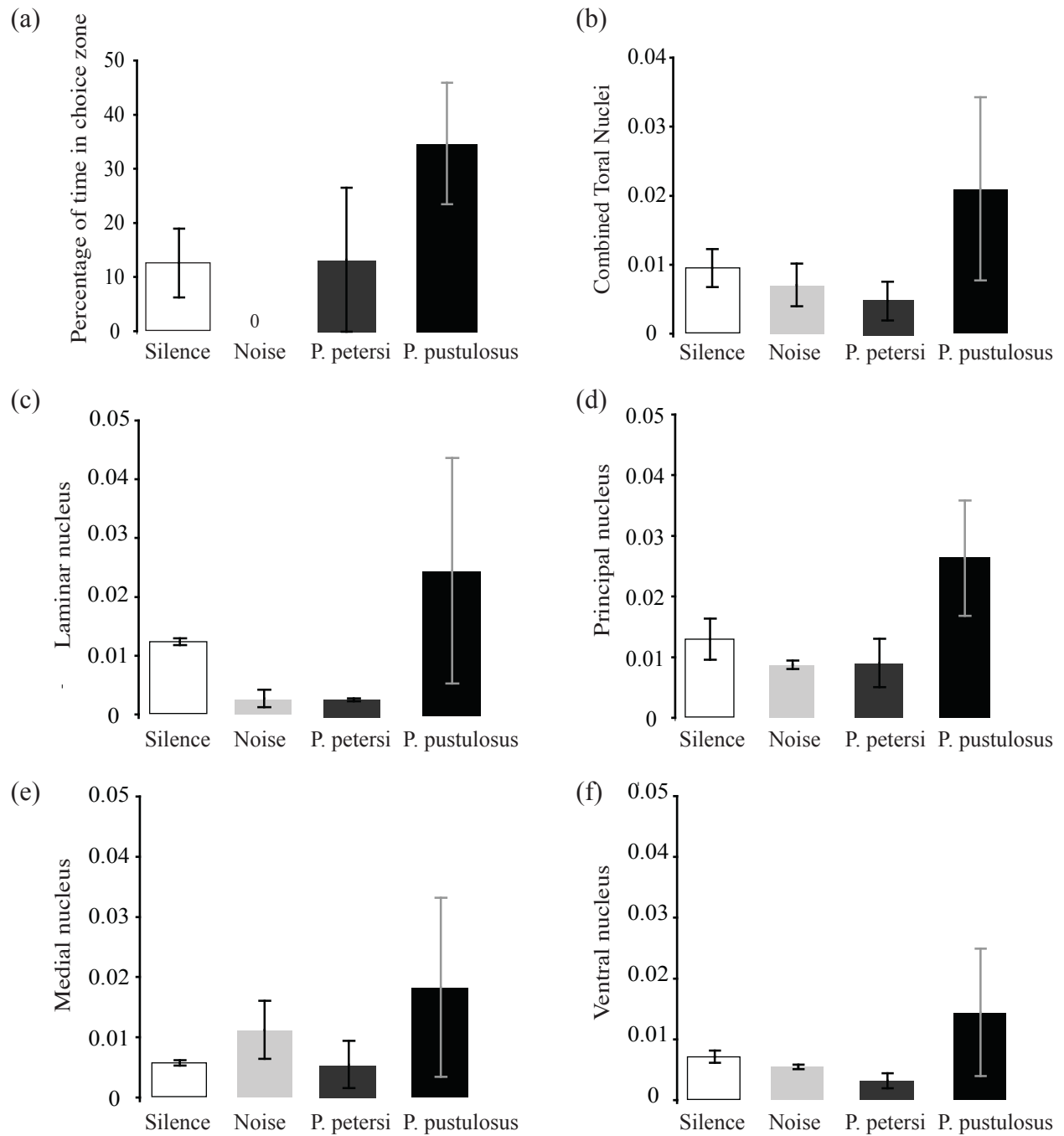
**Figure 2.** (a) Coronal section of torus semicircularis with subdivision boundaries outlined (L: laminar, P: principal, V: ventral, M: medial; OT: optic tectum, AD: anterodorsal tegmentum). Scale bar 0.1 mm. (b). High magnification showing silver grains over toral cell bodies. Scale bar 0.01 mm.



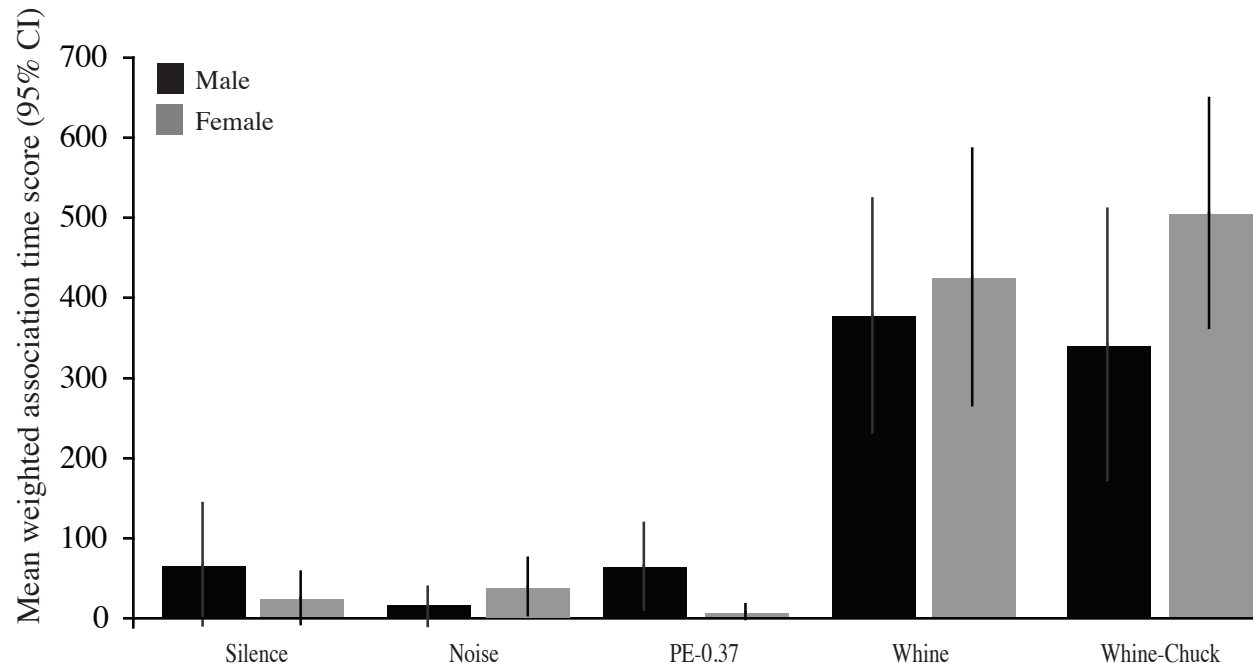
**Figure 3.** Developmental timeline of study that shows the six time points evaluated during postmetamorphic development in lab-reared frogs ( $N = 42$ ) and an identical battery of tests conducted on field-caught, reproductive adults ( $N = 24$ ). At each time point the six tests depicted were conducted and five behavioral measurements were recorded in each, resulting in a total of 1,932 trials. Additionally, three two-choice tests were conducted in 2008 on young (approx. time point one and two) juveniles ( $N = 20$ ; 10 males and 10 females; 60 choices). Lastly, hCG injected juveniles ( $N = 13$ ) were tested in three back-to-back whine versus reverse whine tests.



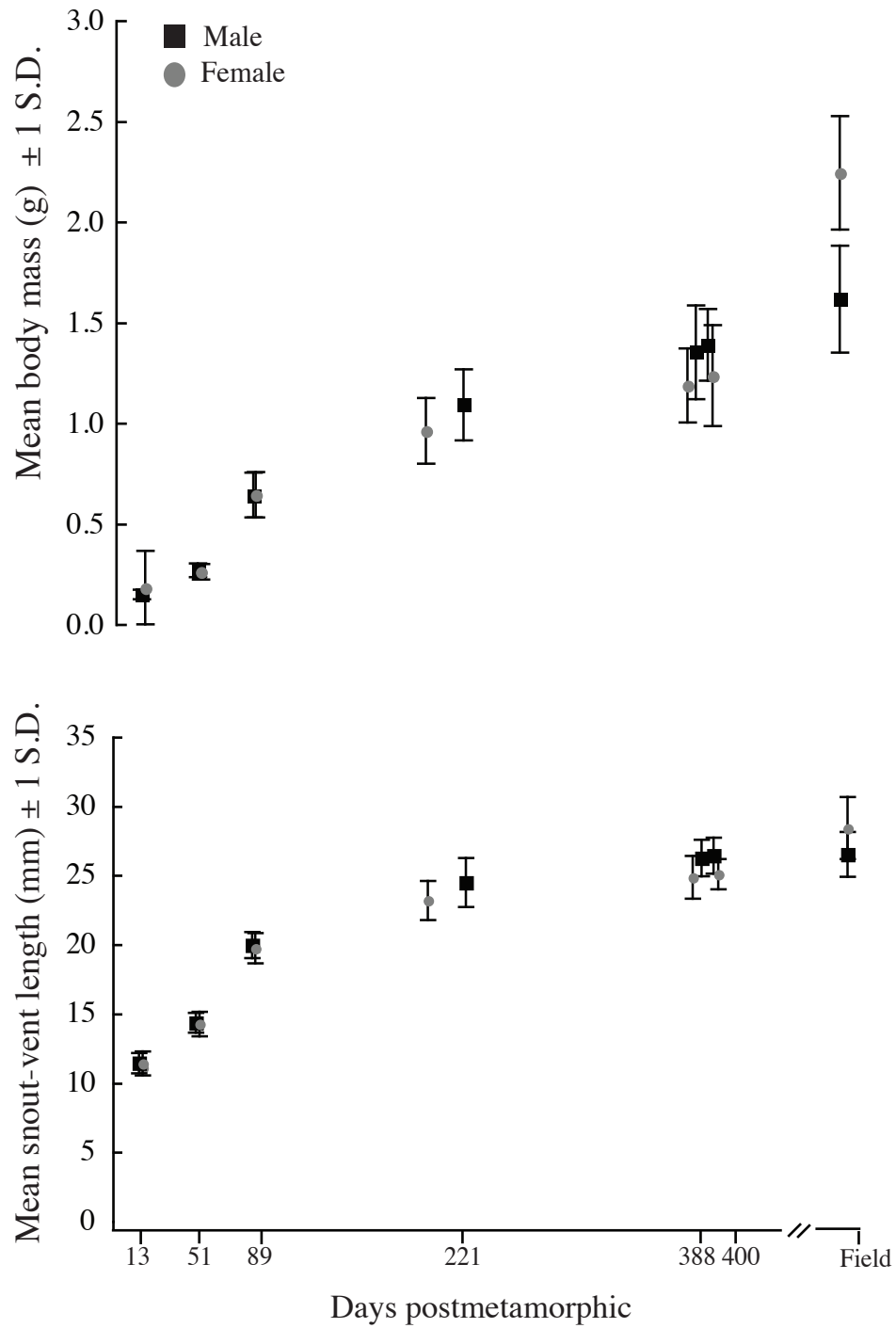
**Figure 4.** Phonotaxis arena showing weighted association time zones (large arc dash), choice zone (small rectangular dash), release point, and dimensions. For simplicity I show only the zones on one half of the chamber but a symmetrical outline was used at the opposite pole.



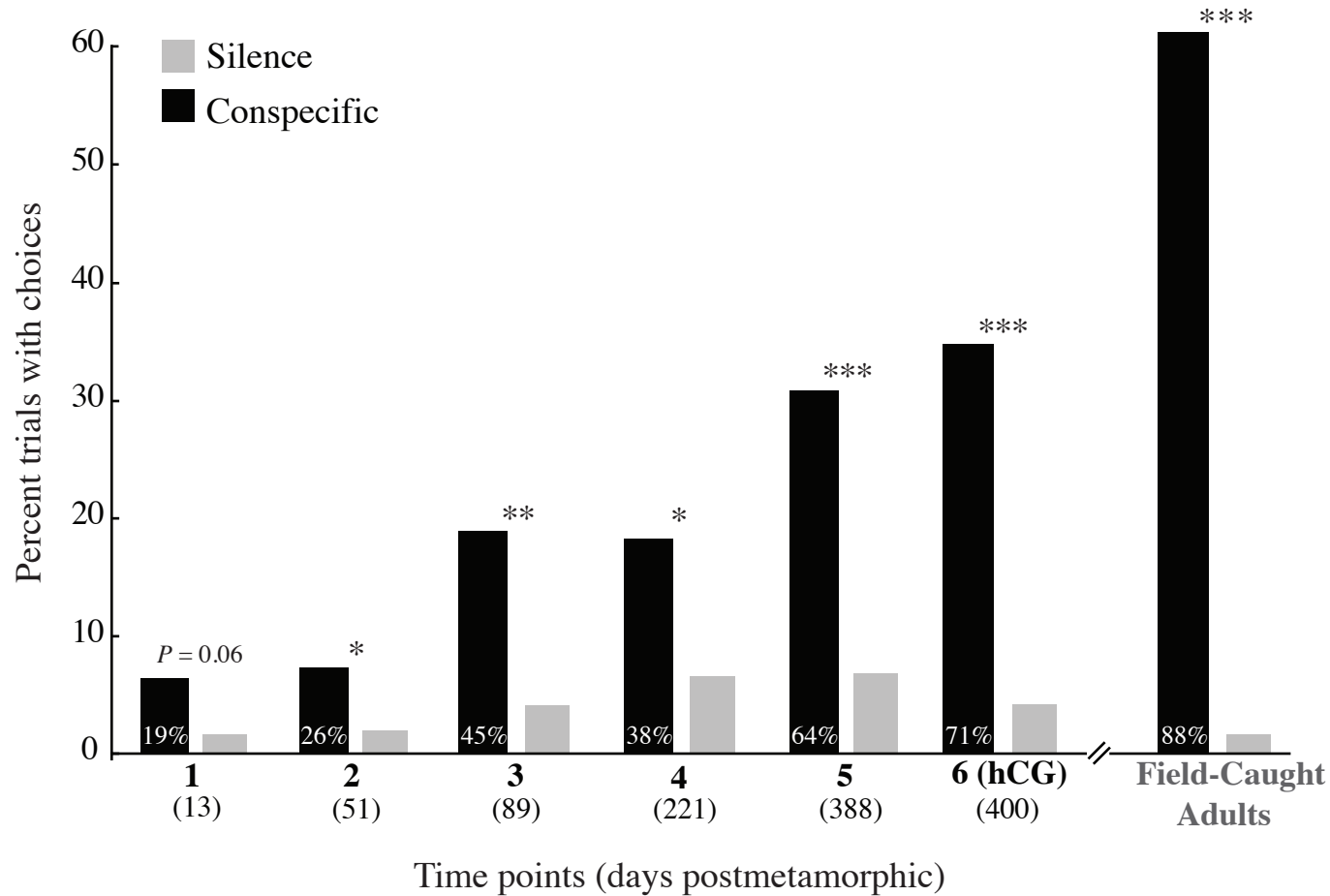
**Figure 5.** Behavioral and in situ results for Experiment 1 (Ch. 2); *P. pustulosus* ( $N = 6$ ), *P. petersi* ( $N = 3$ ), Noise ( $N = 2$ ), Silence ( $N = 2$ ). (a) Percent of time in choice zone  $\pm$  SEM. (b–f) Silver Grain Density (silver grains/pixel of cell area)  $\pm$  SEM.



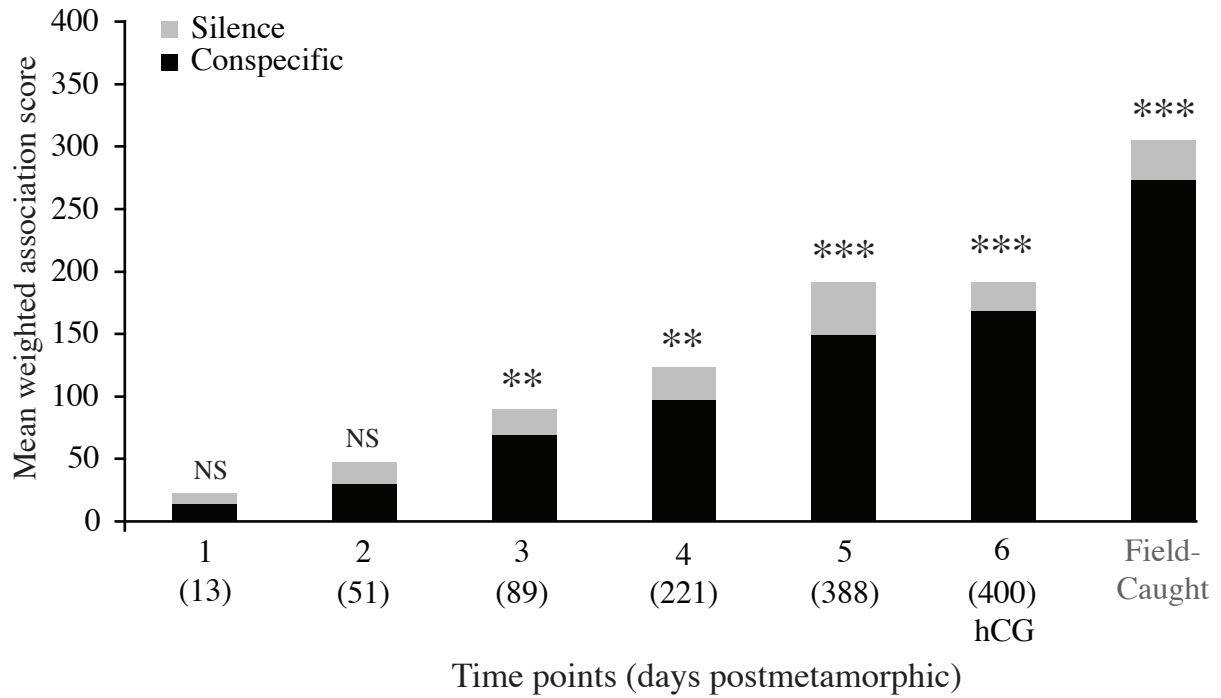
**Figure 6.** Mean weighted association time scores for field-collected adult males and females for the five one choice test conditions.



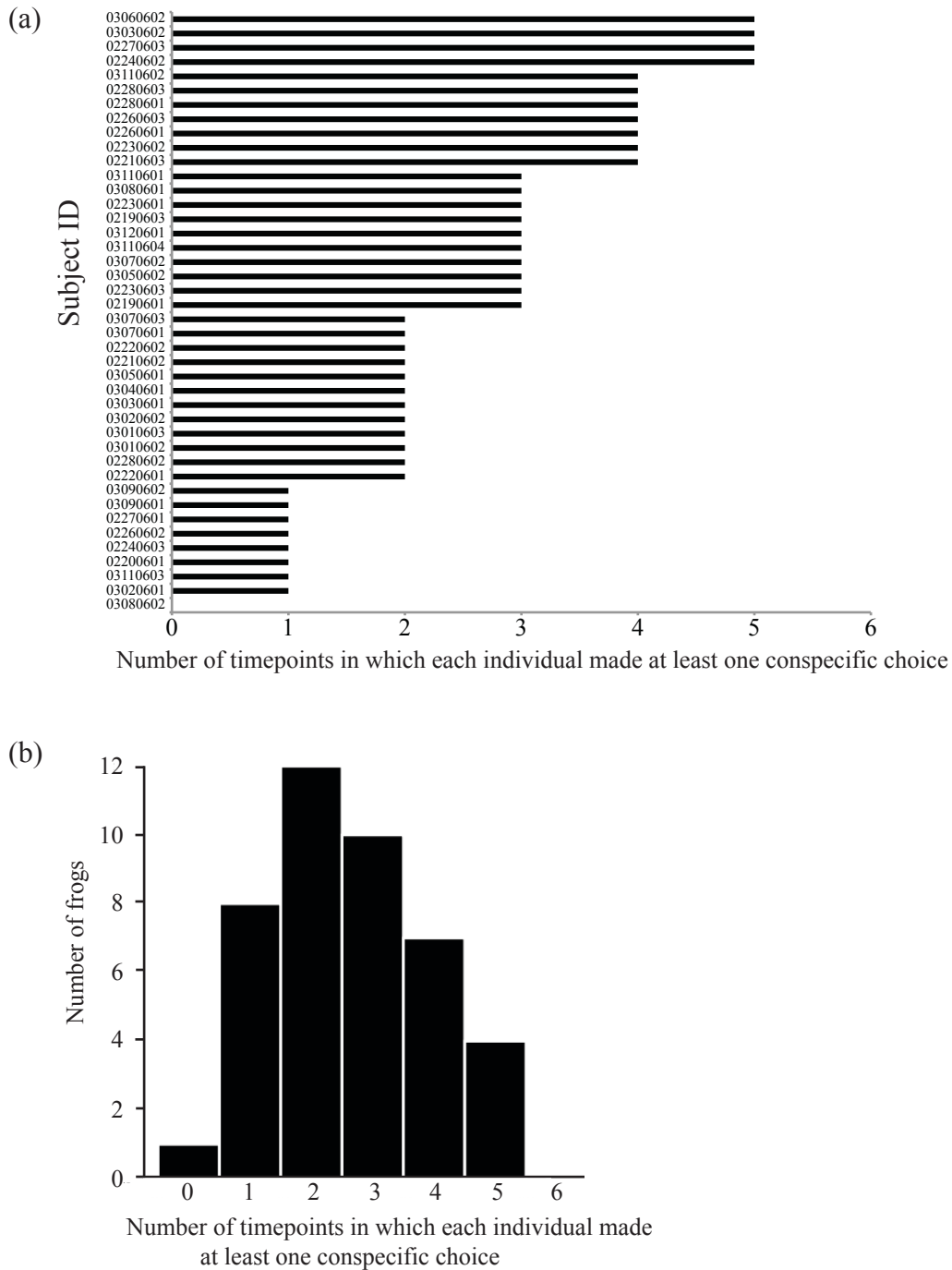
**Figure 7.** Mean body mass and SVL during development. Error bars represent  $\pm 1$  standard deviation.



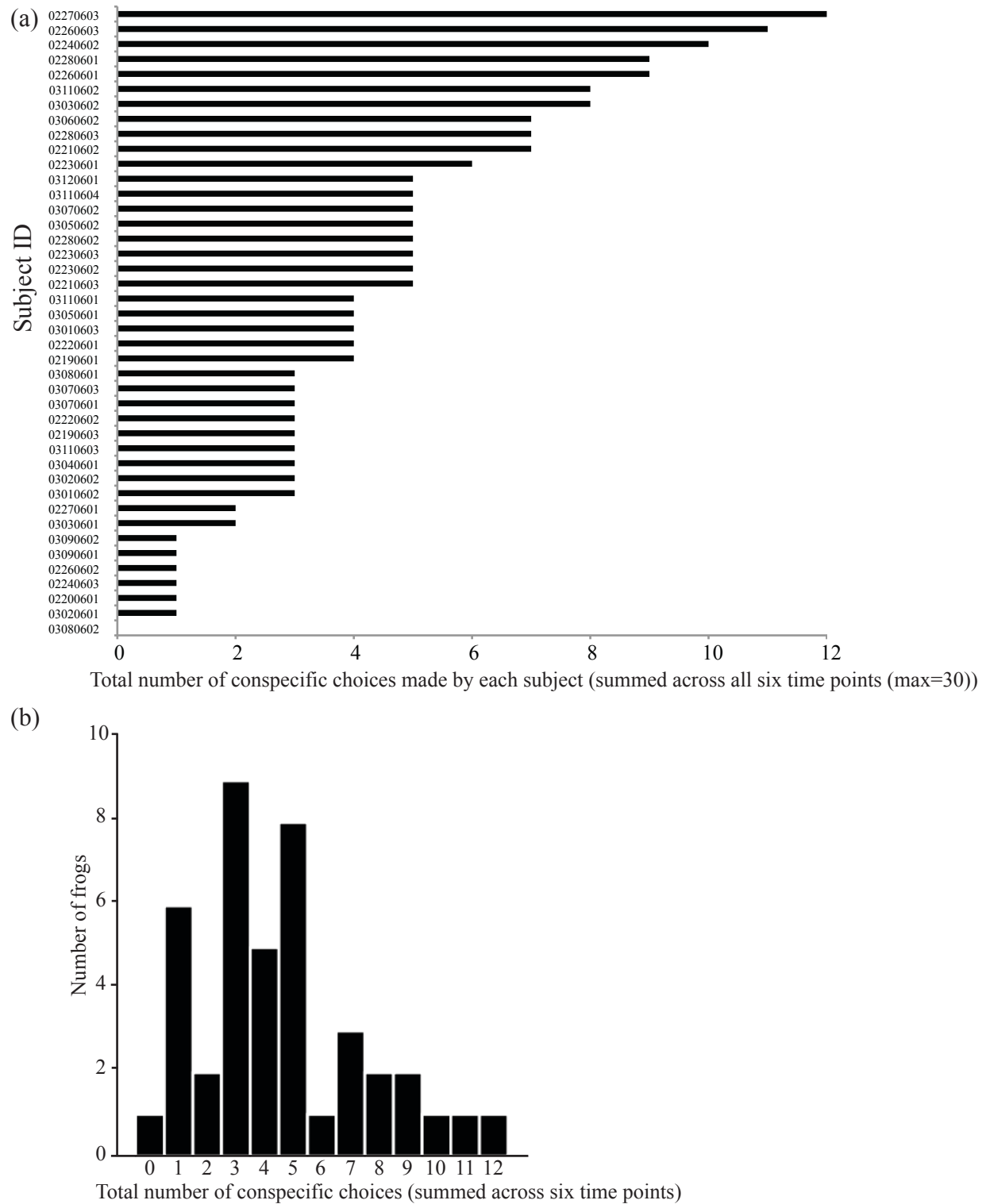
**Figure 8.** The number of phonotactic choices made by males and females during development. A significant preference for conspecific compared to the silent control was observed beginning at time point two. The frequency of choices (bar height) increased gradually during postmetamorphic development reaching a maximum in the reproductive adult state following the injection of hCG. Percentage values inside bars indicate the fraction of animals that made at least one conspecific choice (out of five opportunities) at each time point. Sample sizes were not equal between the lab reared animals ( $N = 42$ ) and field-caught animals ( $N = 24$ ; bar at far right). Significance key: NS = Not Significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



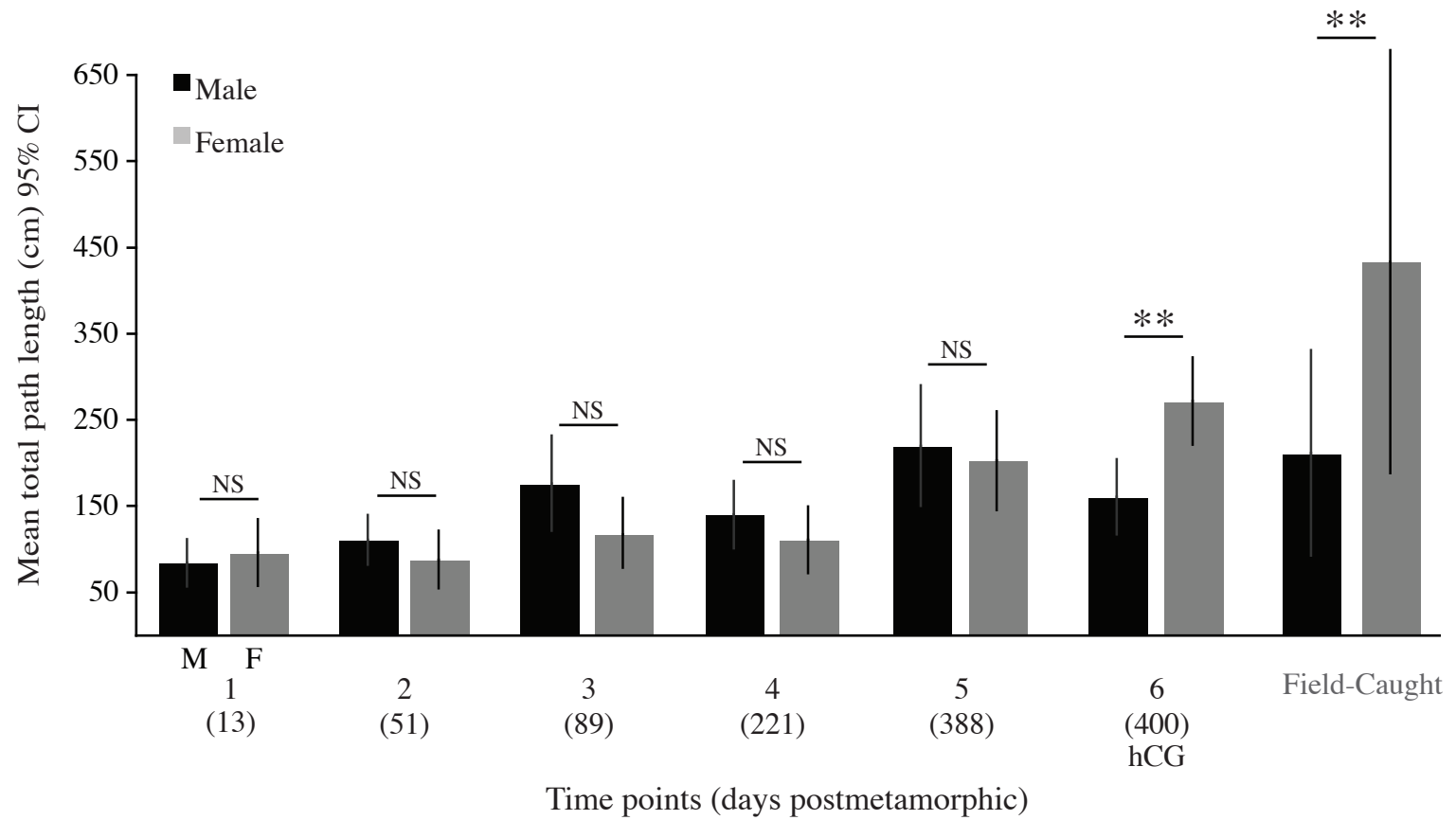
**Figure 9.** Mean weighted association time data for developing males and females. Significance tests for time point one through six were carried out with pairwise comparisons within a repeated measures ANOVA. For field-caught animals, a paired t-test was conducted. Significance key: NS = Not Significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



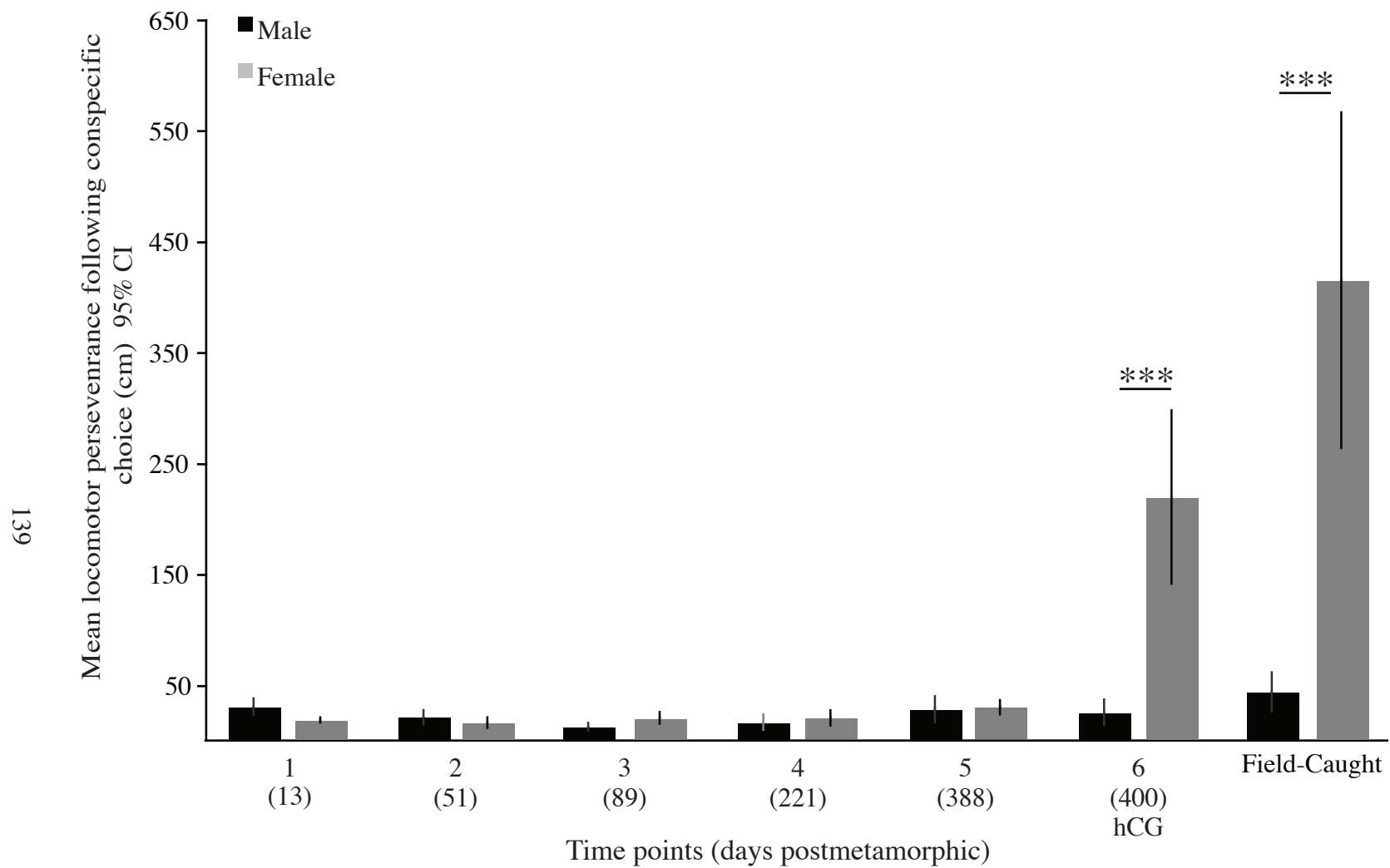
**Figure 10.** Individual variation in presence of conspecific choice behavior during development. (a) Number of time points in which each individual subject made at least one conspecific choice, and (b) the same data plotted as a histogram showing the number of subjects (ordinate axis) that responded with at least one conspecific choice between one and six total time points (inclusive, abscissa); the greatest number of subjects responded in two out of six time points.



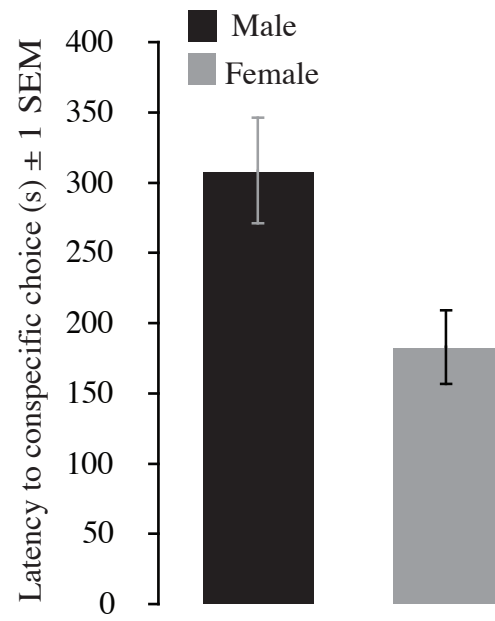
**Figure 11.** Individual variation in degree of conspecific choice behavior during development. (a) Total number of conspecific choices performed by each individual subject summed across all six time points, and (b) the same data plotted as a histogram.



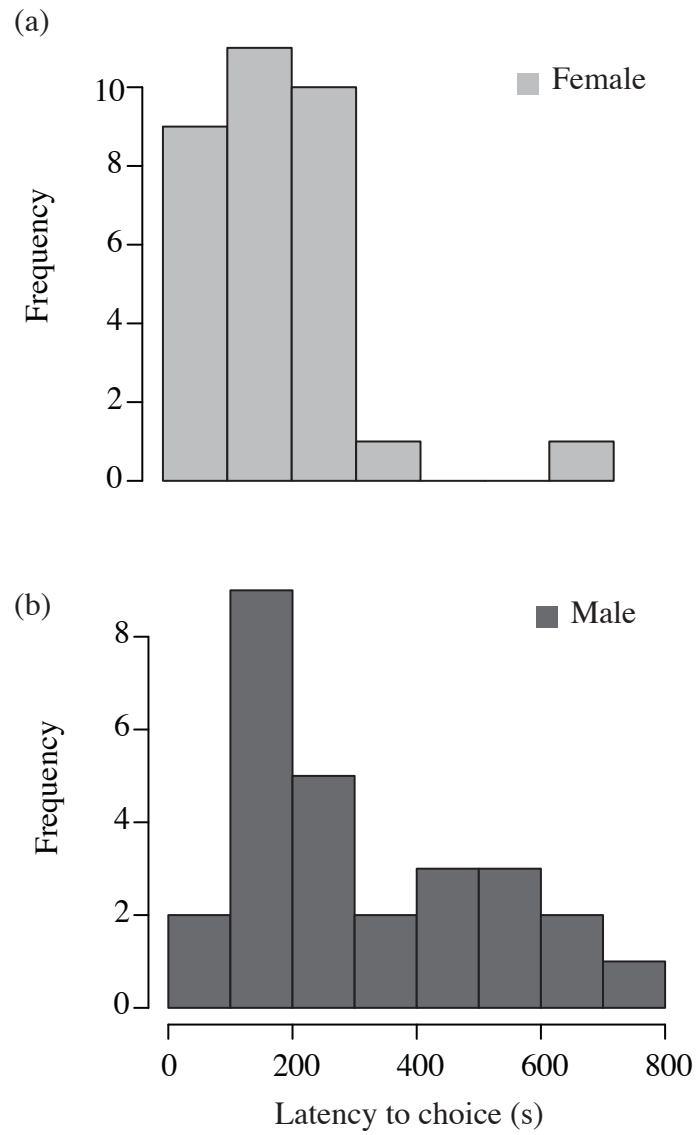
**Figure 12.** Mean total path length for males and females during development comined across all seven acoustic conditions.



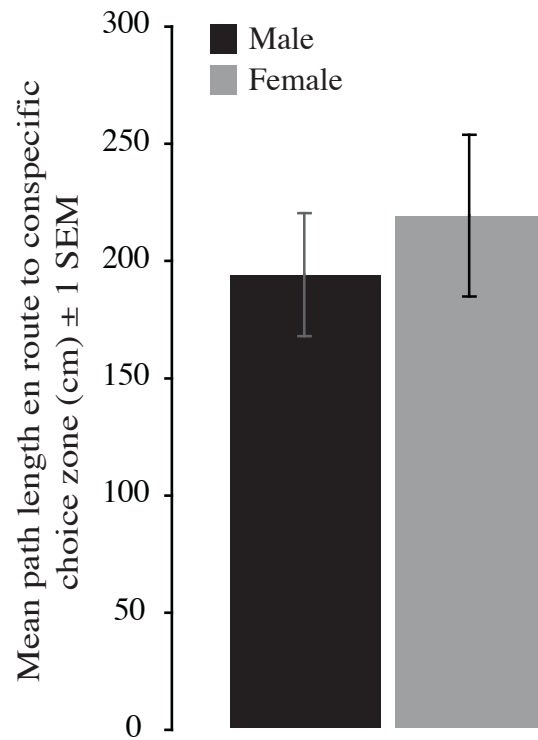
**Figure 13.** Mean path length for males and females inside conspecific choice zones (perseverance) after a choice was made.



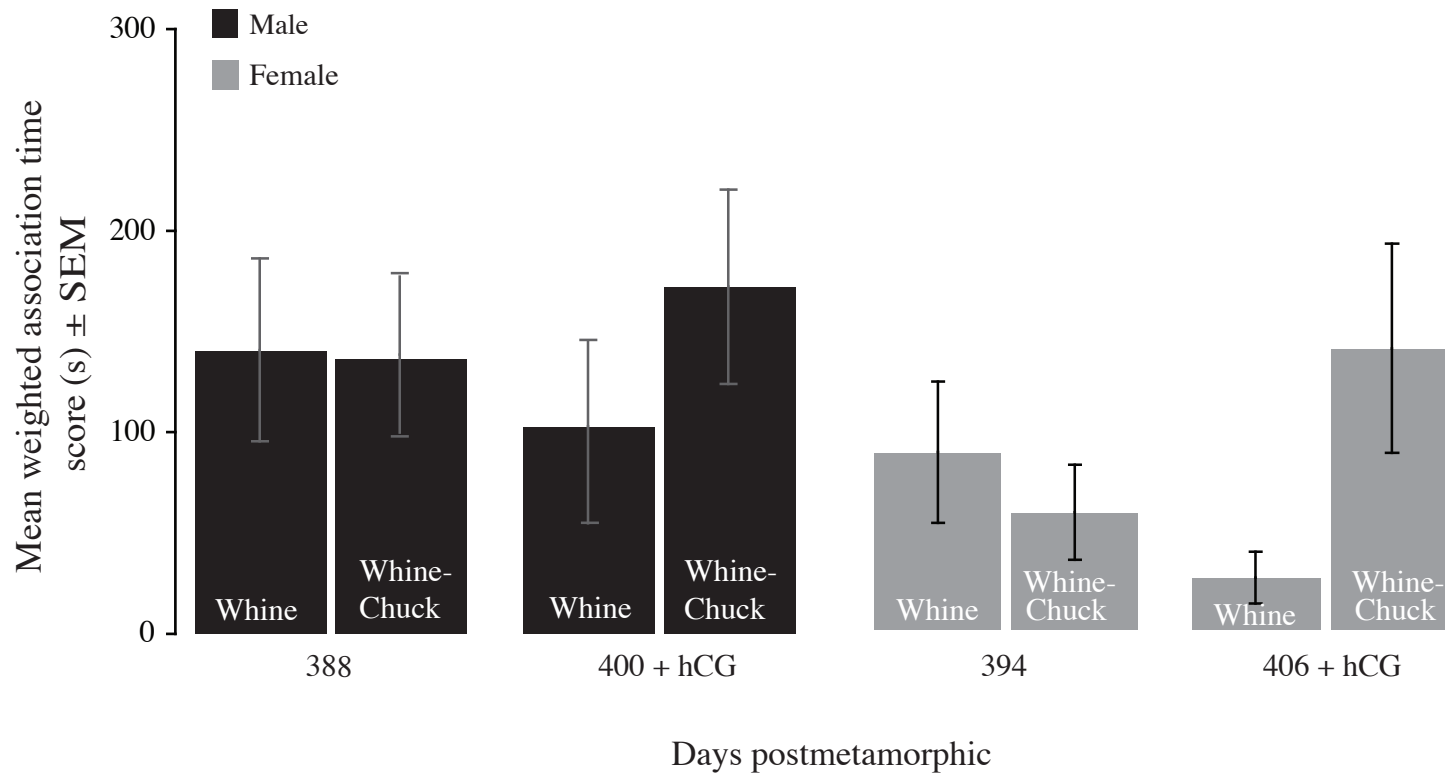
**Figure 14.** Mean latency to choice for field-collected adults. Females had significantly shorter latencies ( $t = 3.27$ ,  $P < 0.01$ ).



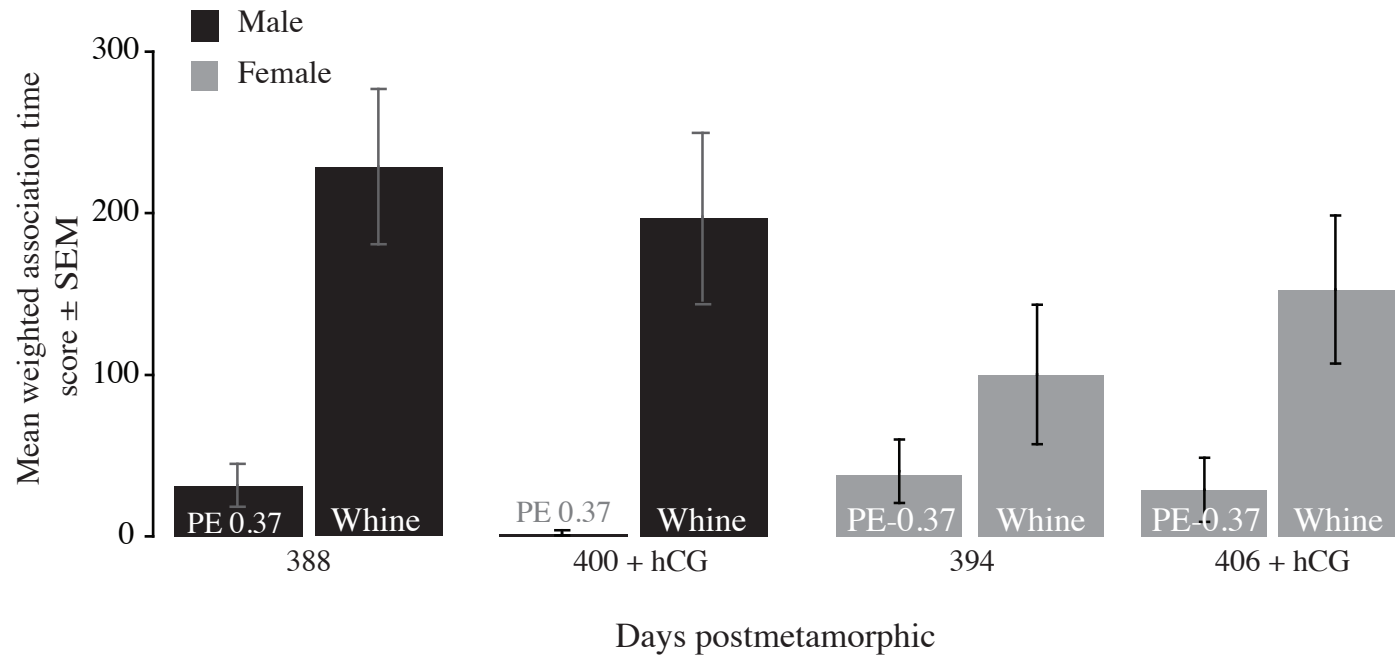
**Figure 15.** Latencies to choice for field-collected (a) females and (b) males. These two distributions differed significantly ( $D = 0.366$ ,  $P < 0.05$ ).



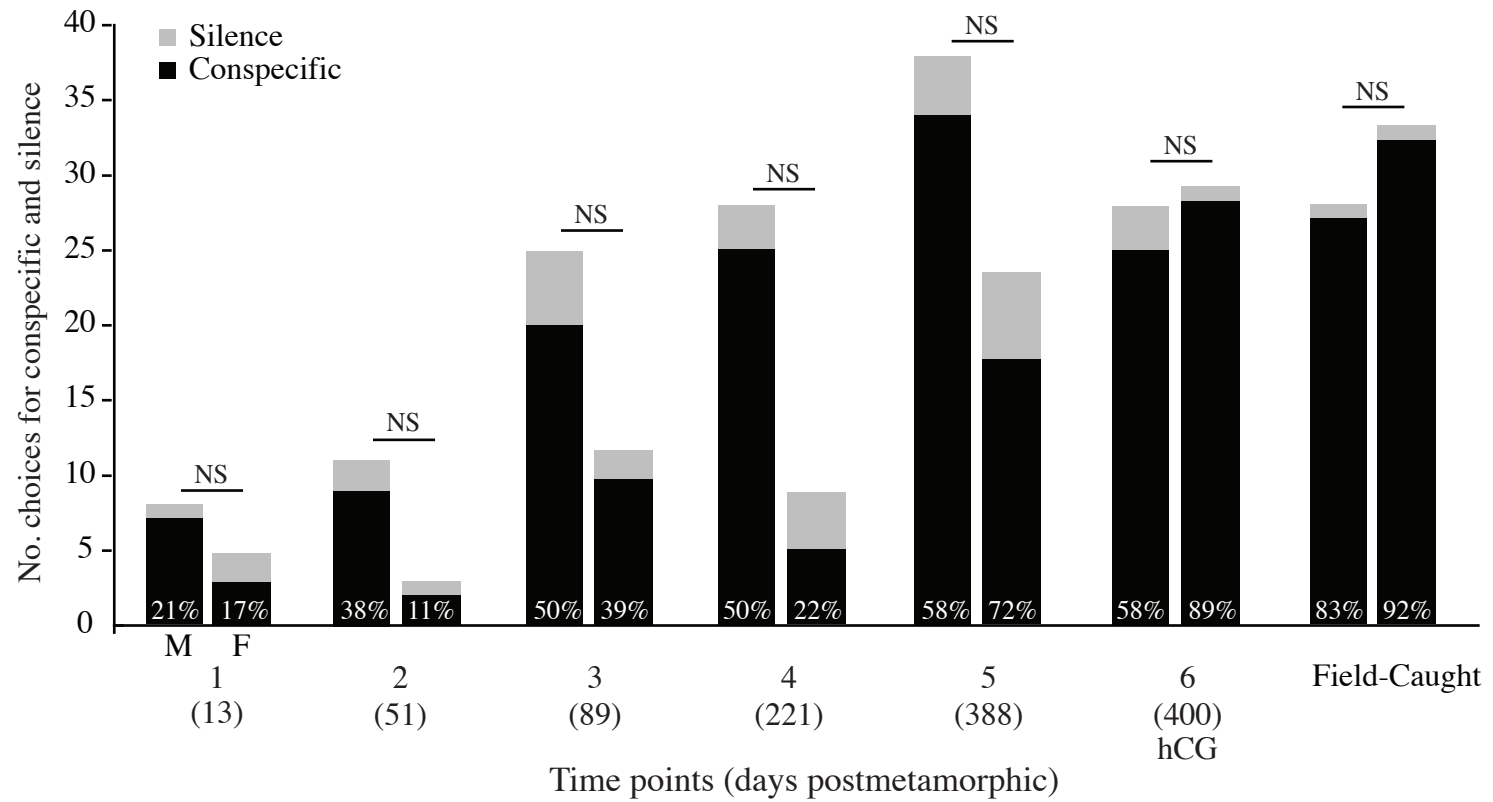
**Figure 16.** Path length to choice zone (excludes path length inside choice zone). No difference was observed ( $t(22) = 0.58$ ,  $P = 0.57$ ).



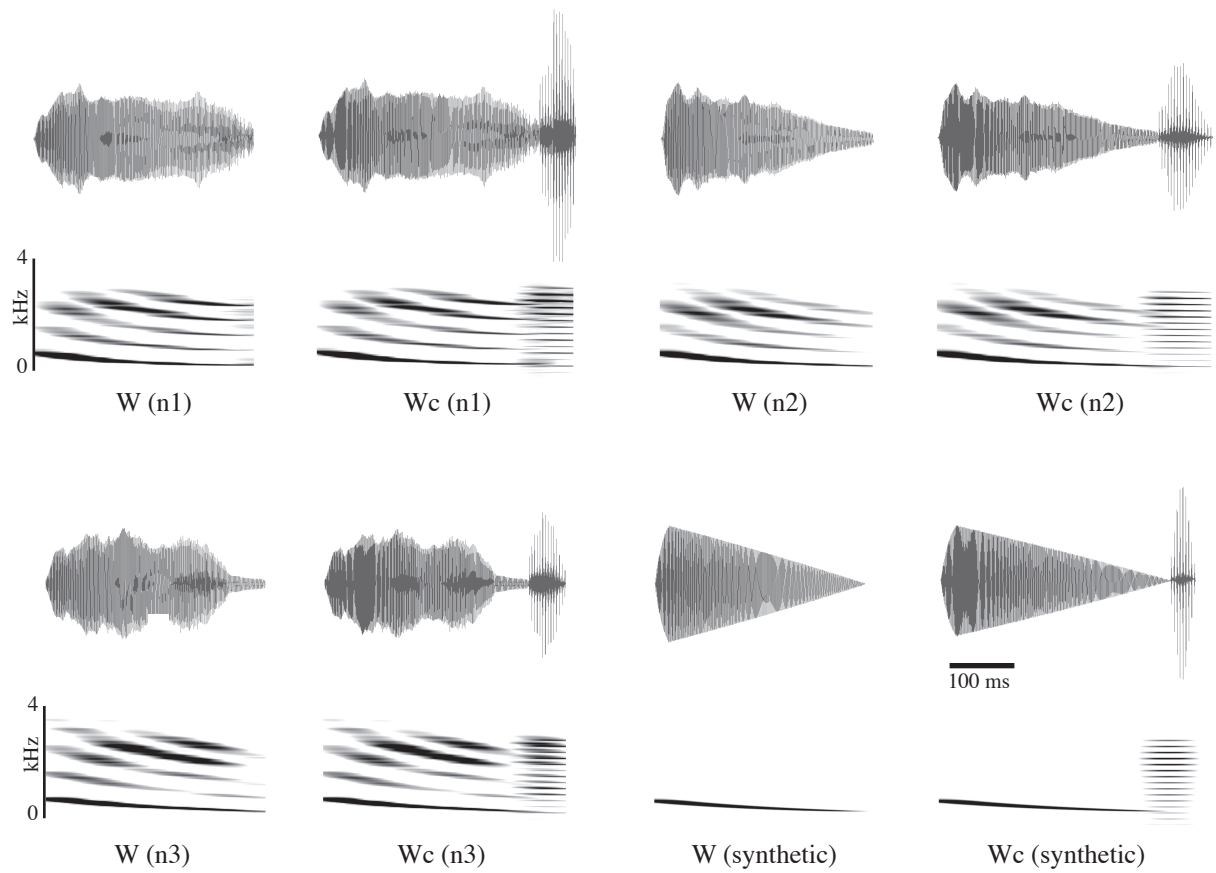
**Figure 17.** Intraspecific preference for lab-reared males at pre- and post-hCG injection time points as assayed by weighted association time. There was not a preference at 388 days or following injection at 400 days. Intraspecific preference for lab-reared females at pre- and post-hCG injection time points as assayed by weighted association time. There was no preference at 388 days but a marginal preference was present following injection at 400 days.



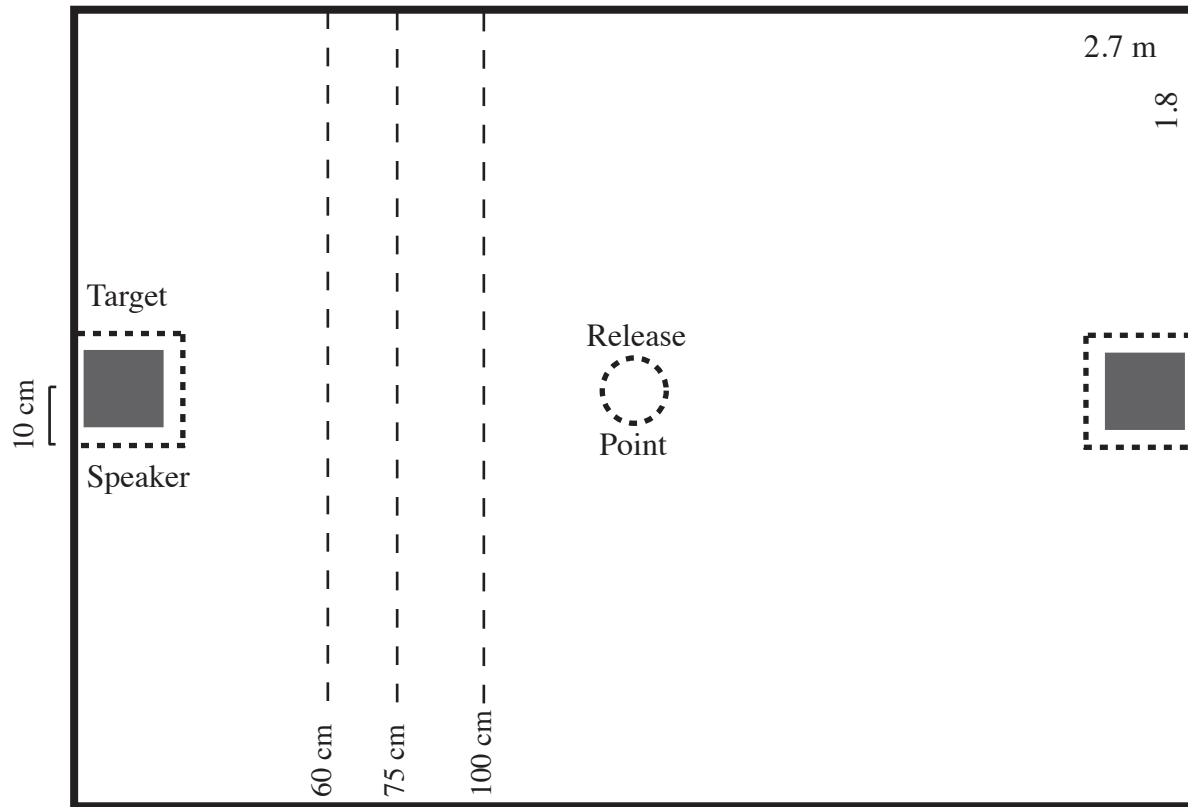
**Figure 18.** Interspecific preference for lab-reared males at pre- and post-hCG injection time points as assayed by weighted association time. There was a preference at 388 days and following injection at 400 days. Interspecific preference for lab-reared females at pre- and post-hCG injection time points as assayed by weighted association time. There was a preference at 388 days and following injection at 400 days. Preference was not affected by hCG treatment in either sex.



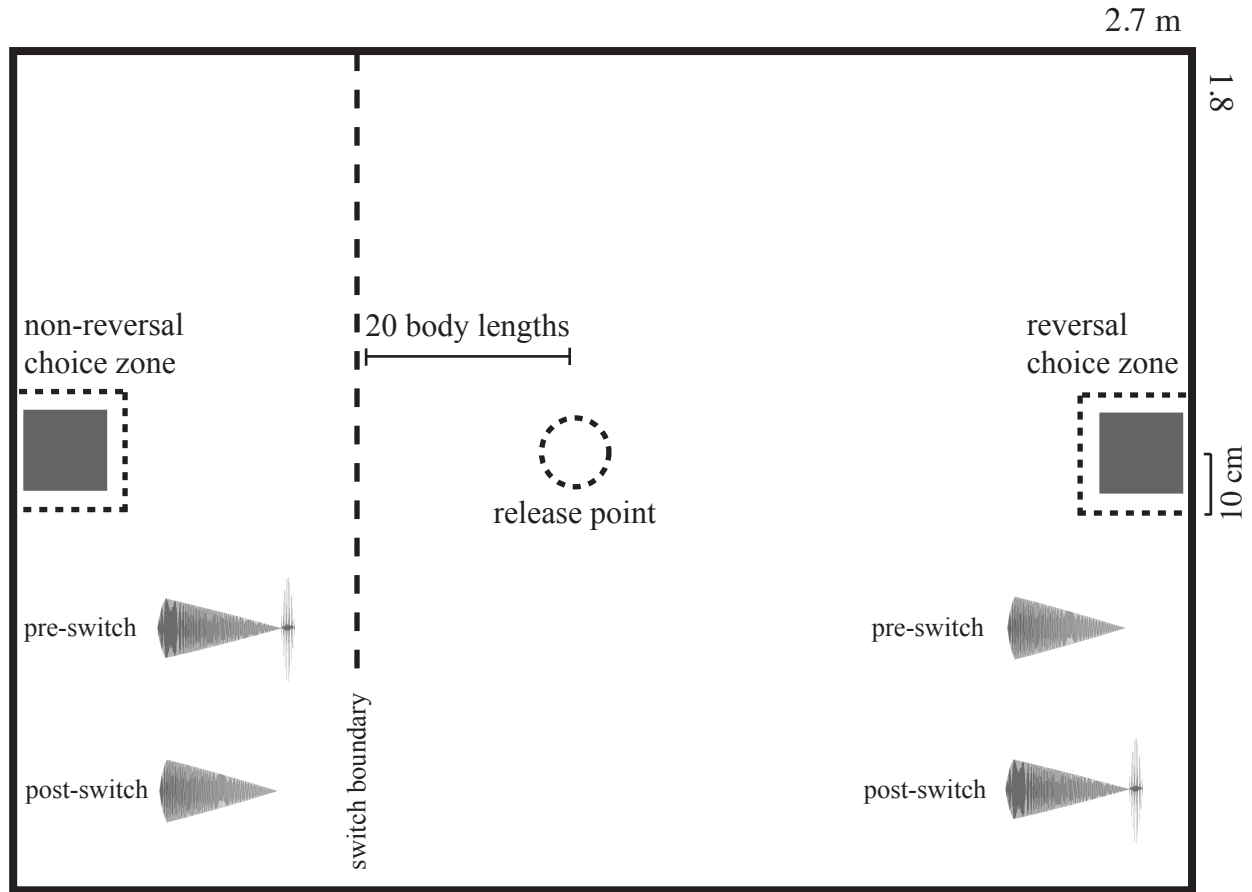
**Figure 19.** Conspecific and silent control choices for males and females did not differ at any time point during development.



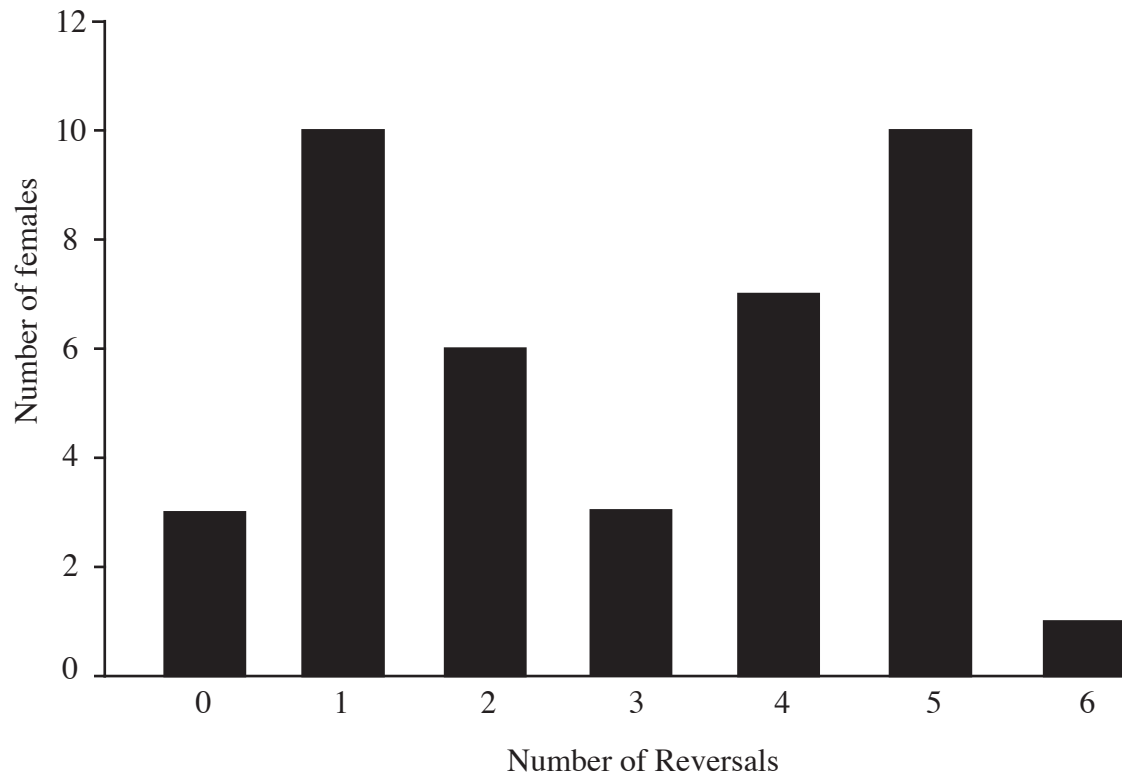
**Figure 20.** Oscillograms (top) and spectrograms (bottom) of 8 stimuli used in this study. The whine with three chucks is not shown but was the same as whine-chuck with 2 identical chucks appended with an interchuck interval of 4 ms.



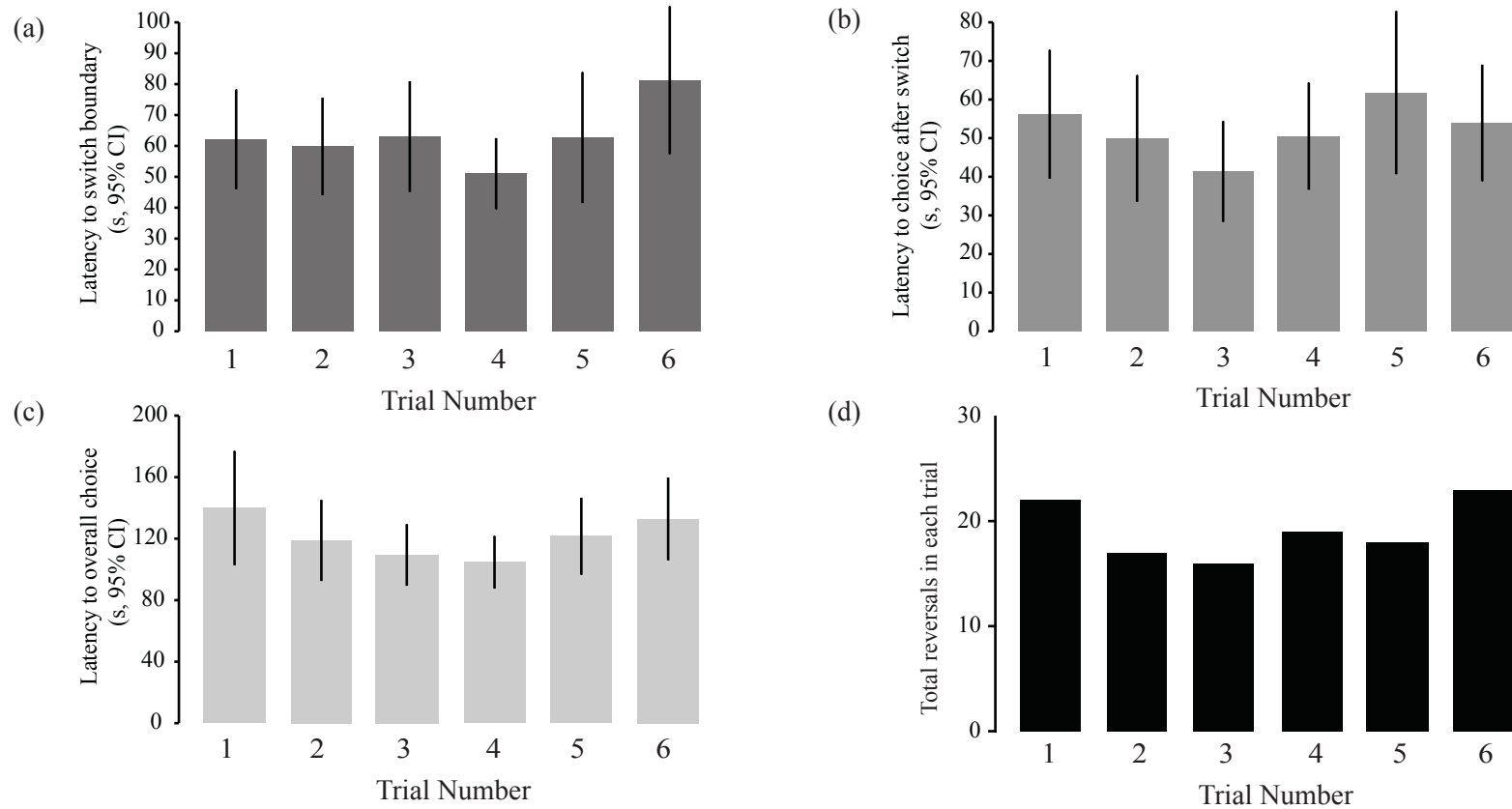
**Figure 21.** Phonotaxis arena including the three decision boundaries and dimensions. A symmetrical placement of these boundaries was present but not shown here for simplicity.



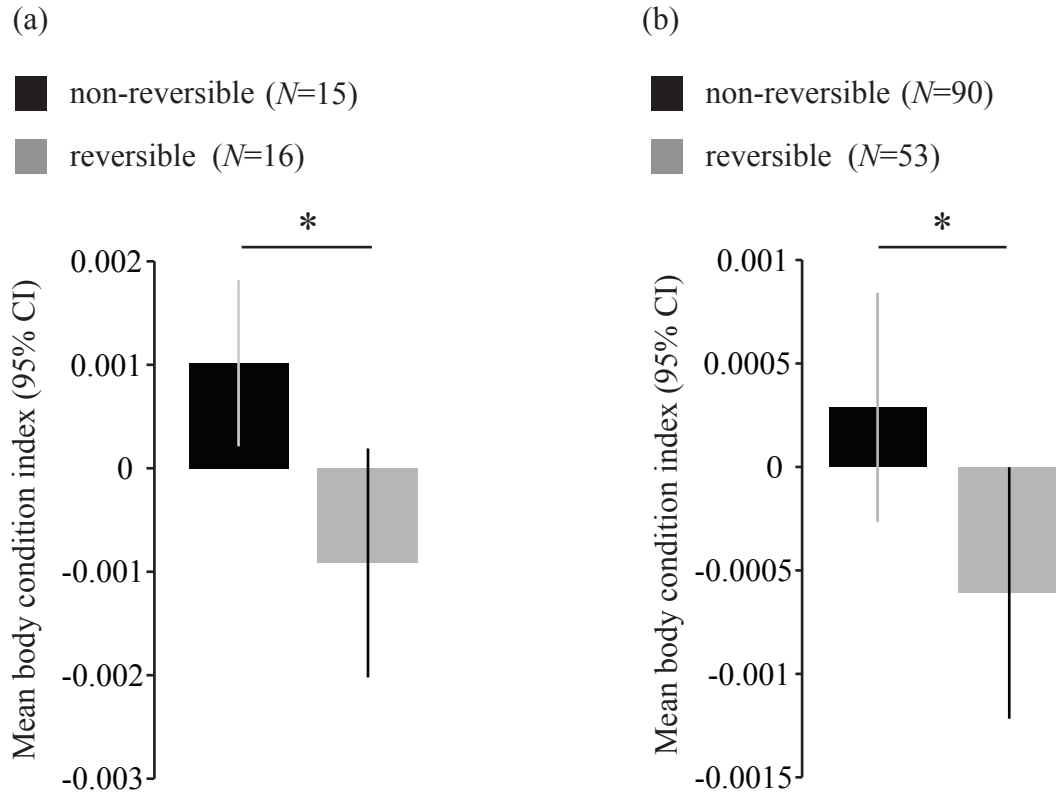
**Figure 22.** Phonotaxis chamber showing arena dimensions and position of release point (midpoint of chamber), speakers, choice zones, and switching boundary (75 cm from speaker, ca. 20 body lengths from release point). This figure depicts one of two symmetrical configurations; between trials the position of call types was swapped to avoid side bias.



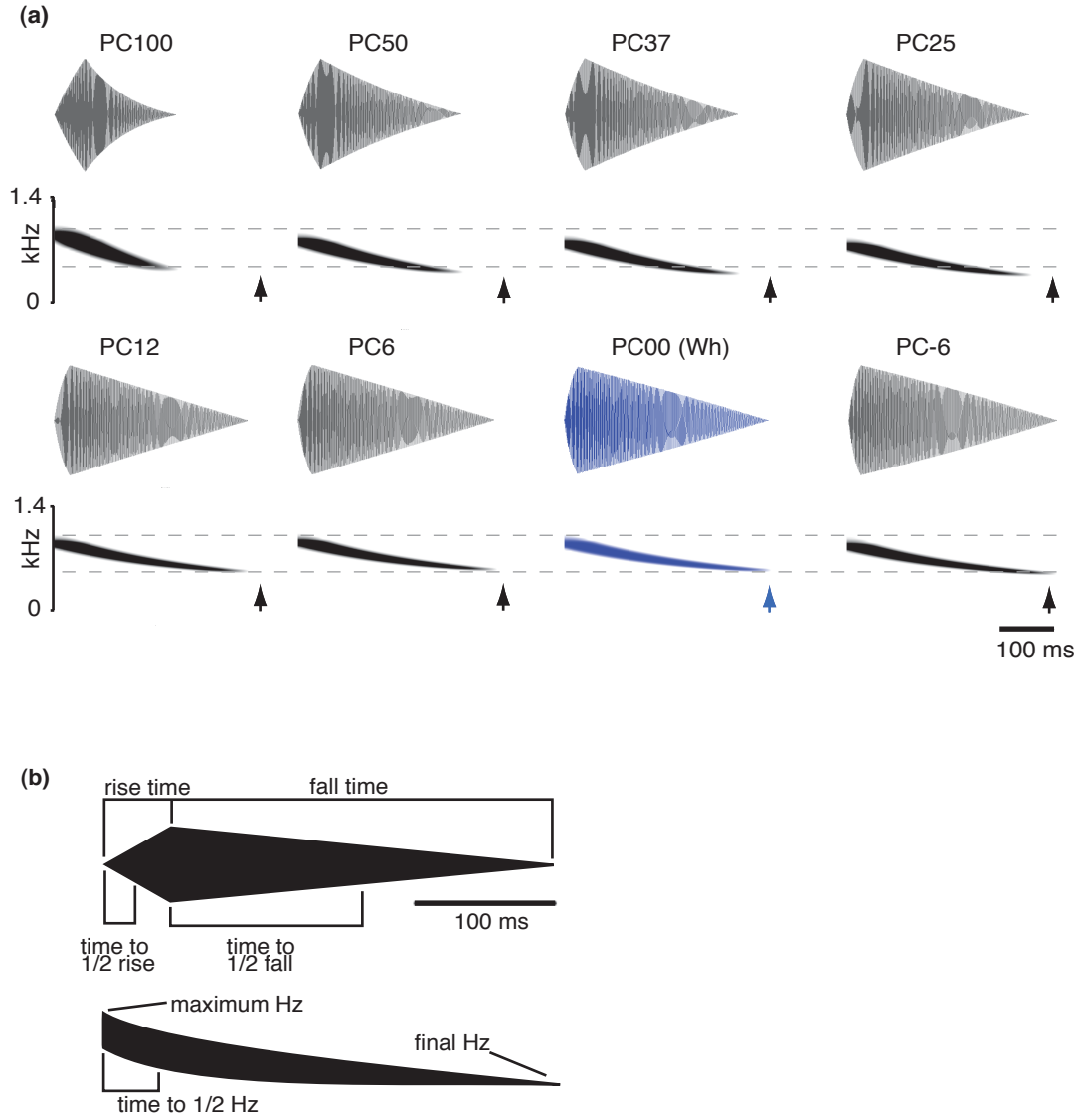
**Figure 23.** Histogram of number of reversals (out of six) for each female ( $N = 40$ ) suggested a bimodal distribution of females in the study population. The population reversal frequency was 47.9%.



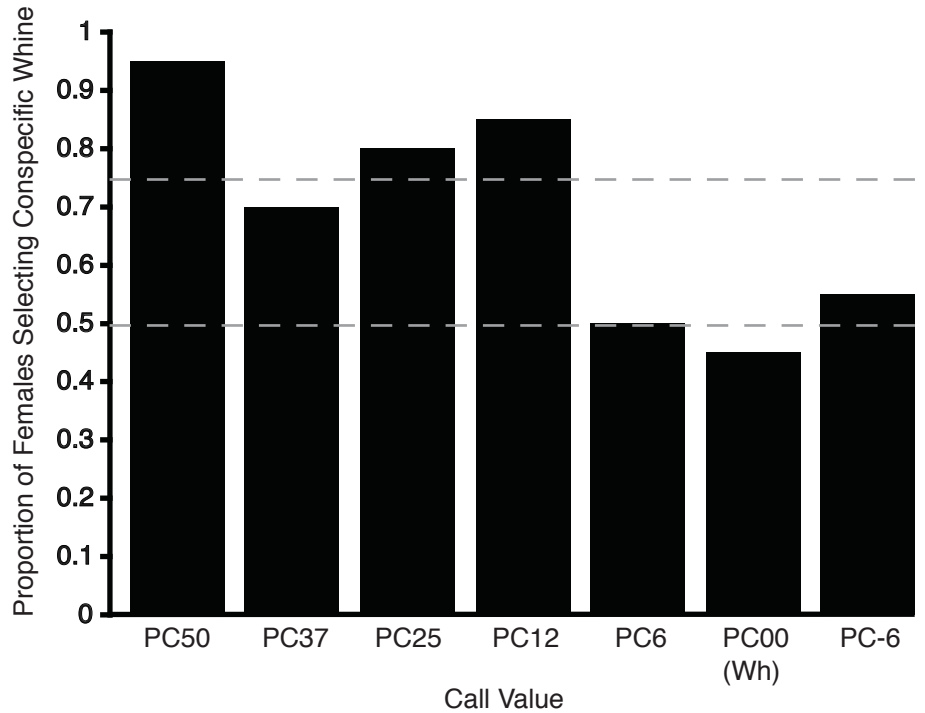
**Figure 24.** The main effect of trial number on latency in a repeated measures MANOVA was not significant ( $F(11,22) = 1.116$ ,  $P = 0.395$ ,  $N = 33$  females, 198 trials). (a) Mean latency to the stimulus switching boundary for each of the six repeated trials was not significantly influenced by trial number  $F = 1.974$ ,  $P = 0.012$ ). (b) Mean latency to choice after stimulus switching for each of the six repeated trials was not significantly influenced by trial number  $F = 1.133$ ,  $P = 0.344$ ). (c) Mean latency to overall choice (from release to choice) for each of the six repeated trials was not significantly influenced by trial number ( $F = 1.979$ ,  $P = 0.105$ ). (d) Total number of reversals performed on each of the repeated six trials. The number of reversals was not significantly influenced by trial number (Cochran's  $Q(5) = 5.04$ ,  $P = 0.41$ ,  $N = 40$ ).



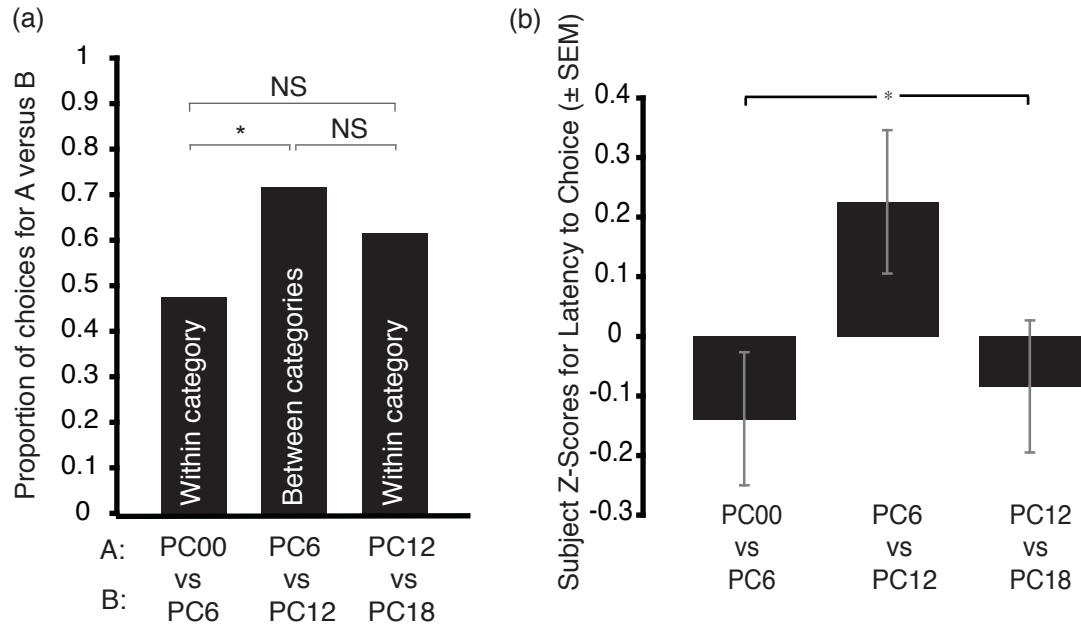
**Figure 25.** Mean body condition ( $\pm$  95% CI) and female reversibility. Females with lower body condition were more likely to be reversible both in (a) Experiment 1 wherein females were characterized as committed or uncommitted following six trials ( $F(1,29) = 7.703$ ,  $P = 0.010$ ), and (b) in an independent study wherein females were characterized following two trials ( $F(1,141) = 4.177$ ,  $P = 0.043$ ).



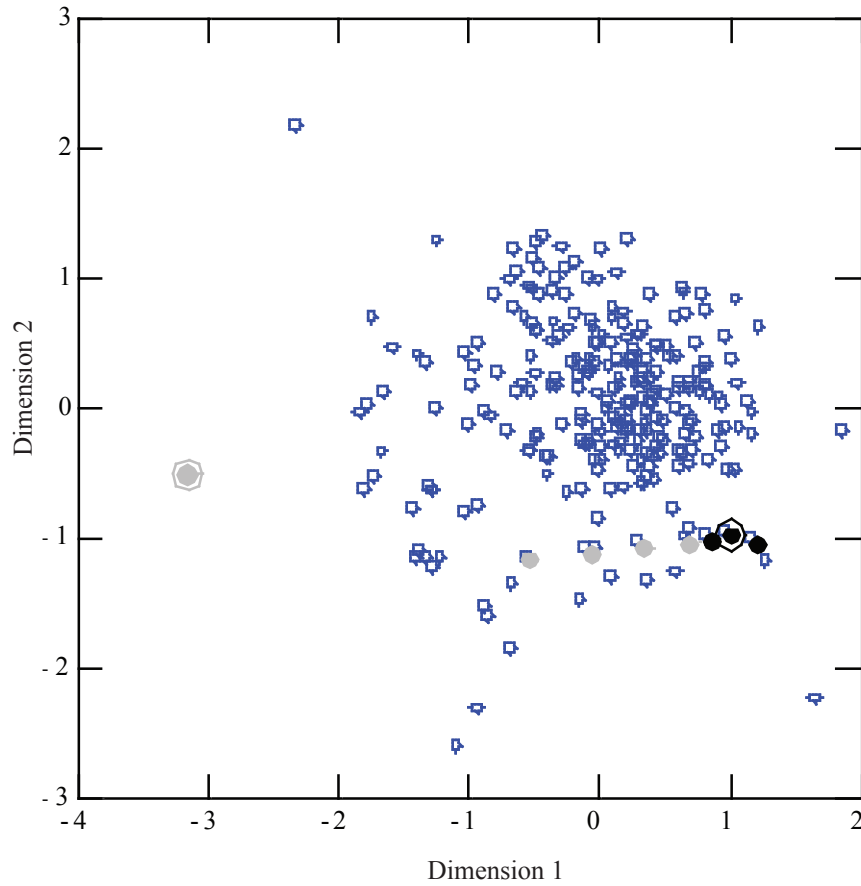
**Figure 26.** (a) Oscillograms and spectrograms of the seven synthetic stimuli used in the labeling component of this study and the heterospecific call (PC100) presented for comparison. Dashed lines indicate the beginning and ending frequencies of the conspecific call (PC00, blue) and arrowheads indicate the duration of the PC00; (b) A stylized oscillogram (top) and spectrogram (bottom) of the synthetic túngara whine are shown along with the seven acoustic parameters used to construct stimuli in this study.



**Figure 27.** The whine (call value 00) is the túngara frog call. PC calls vary in the percentage (e.g., 6, 12, 18%) in which they differ from the conspecific call relative to the call of *P. coloradum*. The proportion of females ( $N = 20$  for each experiment) that prefer the conspecific call to each call variant shows that females label the calls PC-6 through PC6 as conspecific (they do not prefer the conspecific call to these variants), and calls PC12 through PC50 as not conspecific (they prefer the conspecific call to each of these variants). The dashed lines indicate the null expectation (bottom) and the critical value for a significant preference (top).



**Figure 28.** (a) Females were tested with pairs of stimuli in which one stimulus (row A on abscissa) is more similar to the conspecific call compared to the alternative (row B). Females do not show a preference between a pair of calls that differ by 6% within the category that is labeled as conspecific (white versus PC6), while they do show a preference between calls that differ by 6% between the categories that are labeled as conspecific and not conspecific (PC6 versus PC12). The difference in the strength of preference between the within- and between-category is statistically significant. There is, however, discrimination between calls that differ by 6% within the not-conspecific category (PC12 versus PC18), but the strength of this discrimination between categories was not statistically significant. (b) The z scores of the latency to respond from the same phonotaxis tests in (a) show that females take significantly more time to make a choice in the between-category comparison than in either of the within-category comparisons.



**Figure 29.** A multiple-dimensional scaling plot of 300 calls from 50 males (open circles; see Ryan et al. 2003) from Gamboa, Panamá, where females for this study were collected. The filled circles are calls from the PC transect with which females were tested (except for the *P. coloradorum* call which is given as a reference. Black-filled circles are calls that females classify as conspecific and gray-filled circles are those that were classified as not conspecific. The circles with halos are the synthetic *P. pustulosus* (black) and *P. coloradorum* (gray) calls. The graph further highlights a general conclusion of the study that similarity of male calls does not simply predict the responses of females.

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## **Vita**

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