

Sexual Selection in the Spring Peeper, *Pseudacris crucifer*, and Multimodal Signaling
in the Túngara frog, *Physalaemus pustulosus*

by

Kyle Wilhite

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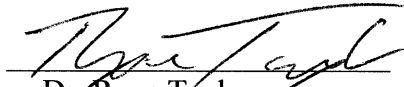
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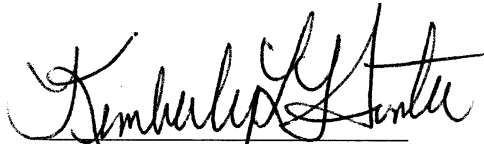
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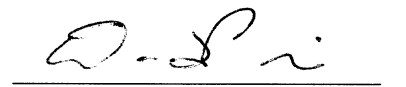
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Abstract

Communication facilitates almost every social interaction between organisms and this communication is especially important for mate acquisition. Anuran amphibians are an excellent model system to study mate choice behaviors and sexual selection. In this work I recorded male calls of the spring peeper, *Pseudacris crucifer*, between two populations (Maryland and Louisiana) and analyzed call parameters such as frequency, duration, and rise and fall time. I found differences in the frequency and the frequency sweep of the calls. I conducted choice experiments to test female mating preferences in the Louisiana population and found a preference for longer calls over short calls. Females did not express a preference for frequency, frequency sweep, or local (LA) versus foreign (MD) calls. I also conducted multimodal signaling experiments on the túngara frog, *Physalaemus pustulosus*. I used natural calls and a robotic túngara frog to determine how females assess different signal modalities (auditory versus visual). I tested previously established attractive calls against unattractive calls, paired with the robo-frog and inflating vocal sac, to see if the visual stimulus would modulate the attractiveness of the call. The presence of the vocal sac did not make the unattractive call more attractive. The vocal sac may have important implications for localization in a more complex environment but here, it did not act as a mate attracting signal. This study provides the ground work necessary for multimodal signaling in spring peepers and for complex sensory environment choice tests in the túngara frog.

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Chapter 1 (Introduction)

Communication is a critical component of the behavioral phenotype of most animal species; it mediates virtually every social interaction among conspecifics as well as many heterospecific interactions (Andersson 1994; Bradbury & Vehrencamp 1998; Maynard et al. 2012; Nava et al. 2012; Roberts et al. 2012; Stange & Ronacher 2012). For example, communication has been shown to influence foraging (Balbuena et al. 2012; Czaczkes & Ratnieks 2012; Golabek et al. 2012), alert social group members about predators (Seyfarth et al. 2010; Thompson & Hare 2010; Casar et al. 2012; Hetrick & Sieving 2012), and influence mate acquisition (Andersson 1994; Brooks & Endler 2001; Ellers & Boggs 2003; Borgia & Keagy 2006; Kirkpatrick et al. 2006; Beckers & Schul 2008; Bee & Micheyl 2008; Baugh & Ryan 2010; Castellano & Gamba 2011). Communication signals can occur through any sensory modality that an animal possesses (i.e., acoustic, visual, olfactory, or tactile), and it is now well understood that most animal communication occurs in multiple modalities simultaneously (Rowe 1999; Hebets & Papaj 2005; Partan & Marler 2005; Gibson & Uetz 2012; Higham & Hebets 2013).

Communication is an integral part of the process of sexual selection, especially as it mediates female mate choice. The processes of natural selection and sexual selection are both driven by individual fitness differences. Unlike natural selection, sexual selection can generate elaborate traits and communication signals that are unlikely to provide the bearer survival benefits (Darwin 1871; Andersson 1994; Halfwerk et al. 2014). Males typically compete for access to female mates by

broadcasting a signal to females or by direct competition with other males. These bouts may consist of vocalizations, visual displays, seismic signals, production of chemical cues, and/or physical combat.

Female mate choice, based on male courtship signals, has been well documented and this process has driven the evolution of some of the most elaborate behaviors and morphologies in the animal kingdom (Andersson 1994). Swordtail fish, *Xiphophorus helleri*, use an elongated caudal fin to attract females (Basolo 1990); bowerbirds, *Ptilonorhynchus violaceus*, decorate their bowers with certain colors that attract females (Borgia & Keagy 2006); gray tree frogs, *Hyla versicolor*, produce vocalizations consisting of trains of pulses, in which the length of the calls are evaluated by females (Welch et al. 1998); and crickets and katydids stridulate their legs or wings to make chirps that females assess (Olvido & Wagner 2004; Beckers & Schul 2008). Likewise, many birds use songs and dances to advertise themselves to females (Searcy 1992; Williams 2001).

Auditory courtship signals that are attractive to females tend to be more energetically expensive to produce (Andersson 1994; Bradbury & Vehrencamp 1998; Hoback & Wagner 2008; Byers et al. 2010). For example, Taylor et al. (2007) showed that squirrel tree-frogs, *Hyla squirella*, prefer faster call rates. Similar preferences have been shown in the Woodhouse's toad, *Bufo woodhousii*, (Sullivan 1983), neotropical treefrogs, *Hyla ebraccata*, (Schwartz & Wells 1984; Schwartz 1986), and gray tree-frogs, (Klump & Gerhardt 1987; Schwartz et al. 2001; Gerhardt & Brooks 2009). More recently, these findings have been shown in the Satin

bowerbird (Patricelli et al. 2006), quacking frog, *Crinia georgiana*, (Smith & Roberts 2003), the European tree-frog, *Hyla arborea*, (Fiedl & Klump 2005), and the sage grouse, *Centrocercus urophasianus*, (Patricelli & Krakauer 2009). These strong female preferences are likely to drive the evolution of male traits, thereby helping to drive the diversity of animal signals (Andersson, 1994).

Multimodal signaling is the communication of signals through more than one sensory modality, and is now known to be common in most communication systems (Partan & Marler 1999). These combined signals can include chemical, auditory, visual, or seismic components (among others). Hebets and Papaj (2005) classified multimodal signals in three categories, content-based signals, efficacy-based signals, and inter-signal interaction. Content-based signals evolved in response to transmitting information. In this case, a complex signal can contain multiple messages, whereby each signal component communicates different information. Conversely, the multiple signals could provide the same information, increasing the probability that the receiver will perceive the information (Hebets & Papaj 2005). Efficacy-based signals combine to increase the efficiency at which a signal is received. One signal could act as a means to garner the receiver's attention, making the second signal easier to perceive. Noisy communication environments are likely to promote the evolution of efficacy-based signals. Here, each signal component acts as a backup to the other when environmental noise is likely to obscure the detection of any particular signal component (Hebets & Papaj 2005). Multiple signals can be processed simultaneously (parallel processing). In the grasshopper, *Chorthippus*

biguttulus, when pattern recognition and directional hearing are accomplished at the same time, reducing the time to elicit a response (Helvesen 1984; Ronacher & Krahe 2000). A multimodal signal could also enhance the neuronal response, through sensory bias for multiple modalities. In a study by Meredith and Stein (1996), visual and auditory neurons overlap 85% in the cat and a multimodal stimulus increased the multisensory response by 148%. The last category, inter-signal interaction, occurs when multisensory signals combine to influence receivers in unpredictable ways. Thus, the production of one signal alters the ability to produce another signal or changes a receiver's response to a second signal (Hebets & Papaj 2005). Signals can act as amplifiers where one signal serves to make another signal more conspicuous. Male *Anolis* lizards signal by raising and lowering a dewlap in a variety of signaling contexts (Fleishman 2000). The bright colors on the dewlap increase the receiver's ability to detect the movement. The presence of a signal can also increase the receiver's ability to learn or remember the signal when paired with a second signal. Chicks (*Gallus domesticus*) were trained to find food under hats of two different colors, coupled with the absence or presence of a sound. The sound significantly increased the speed at which chicks were able to avoid hats without food (Rowe 2002).

Anuran amphibians use vocalizations for mate acquisition and territorial defense (Ryan 2001; Gerhardt & Huber 2002). Males gather at small bodies of water, usually an ephemeral pond or creek, and begin vocalizing as a chorus. This chorus attracts females to the water to begin the process of selecting a mate. Male calls can

also serve to space competing males and many species will defend their calling sites against other males (Schwartz 1989). Females arriving at the chorus listen to male vocalizations and choose a mate based primarily on call properties. Females approach calling males and evaluate them based on call characteristics. When she chooses, she bumps him and he clasps her, initiating amplexus. Then the pair moves away from the chorus to lay and fertilize a clutch of eggs.

One aspect of sexual selection in anurans that remains poorly understood is how female choice is expressed in more realistic chorus environments (Schwartz et al. 2001); that is, how do acoustic interference and different signal components interact to influence female mate choice (Smith & Roberts 2003; Taylor et al. 2007; Bee & Micheyl 2008)? Most phonotaxis studies use a simple, two-choice test to assess female mating preferences (Bee 2008; Kostarakos et al. 2008; Bee & Schwartz 2009; Baugh & Ryan 2010; Bonachea & Ryan 2011). Two-choice experiments are excellent for assessing female mate preferences, but rarely in nature does a female frog choose only among two competing males. In this work, I use two-choice tests to characterize mate choice in the spring peeper, *Pseudacris crucifer*, a species that relatively little is known about; this will provide the foundation for future, more complex studies in the spring peeper. The túngara frog on the other hand, is a well-studied system and much is known about female mating preferences in this species. The second goal of my study is to test how female túngara frogs assess calls in more natural chorus settings.

The spring peeper, *Pseudacris crucifer*, is a treefrog that inhabits eastern North America, ranging from Canada to Louisiana and as far west as Minnesota (Forester & Czarowsky 1985; Austin et al. 2002; Moriarty & Cannatella 2004). Males gather at ponds and vocalize from open to semi-protected areas. They can either produce an advertisement or aggressive call. The advertisement call is a simple “peep” that is repeated; the aggressive call is a trill of pulses that is usually produced 1 – 4 times in sequence (personal observation). Peepers increase duration of the aggressive call in the presence of other males (Schwartz 1989). This helps maintain calling territories, but males face a tradeoff between maintaining a territory and attracting a female, as the aggressive call is not preferred by female peepers (Marshall et al. 2003). There is disagreement on whether spring peepers have preferences for certain call properties (Forester & Czarowsky 1985; Schwartz & Gerhardt 1998) and I aim to outline those basic preferences in order to open up the system to multimodal signal testing.

The túngara frog, *Physalaemus pustulosus*, is a neotropical frog that ranges from Southern Mexico to Northern South America (Ryan et al. 1996). The males gather at small puddles and ditches and vocalize while floating in the water. Their calls consist of two parts. The first part is a “whine” followed by one to seven optional “chucks” (Ryan 1980). The whine itself is both necessary and sufficient to elicit a response from females, but adding the chuck increases the whine’s attractiveness five-fold (Gridi-Papp et al. 2006). As in most frog species, male túngara frogs also have a vocal sac that inflates when they call, but their vocal sac is

larger than required for calling efficiency alone (Dudley & Rand 1991). The vocal sac influences female choice (Taylor et al. 2008), and here I examine the role of the vocal sac in mate choice under more realistic (i.e., acoustically noisy) conditions.

Chapter 2 (Spring Peeper Call Recording, Analysis, and Synthesis)

Materials and Methods

Anuran Communication

On late winter/early spring nights, anywhere from a few up to a few hundred male spring peepers gather at dusk and begin producing vocalizations (mating advertisement calls). I recorded spring peeper advertisement calls from choruses and analyzed them to obtain average population values for the call properties. These include the fundamental frequency, the number of harmonics, the dominant harmonic, the frequency throughout the call, the duration of the call, and the rise and fall time of the call. These values permitted the characterization of male calls and were necessary for constructing synthetic calls for phonotaxis experiments.

Study Sites

I collected frogs from two populations. The first was a population in Hebron, MD (38°24'04.65"N, 75°41'27.27"W) on Maryland's eastern shore. This region has a humid temperate climate, and I collected frogs during the breeding season from March – May (2011 – 2012). The Hebron site consists of a drainage ditch where water depths fluctuate between 20 and 60 cm depending on rainfall. If rainfall did not occur for an extended period of time, the ditch dried completely. The ditch is also adjacent to a few hectares of forest.

The second population I sampled, for geographic comparison, was in Louisiana. South Louisiana's climate is warm–temperate. I sampled frogs from three

separate locations separated by a distance of 55 km during the breeding season in January (2011 – 2012). The first location was in Ville Platte (30°39'33.5"N, 92°9'36.11"W) near Highway 103 where a power line right-of-way intersects the road. The low-lying right-of-way lies between two seasonally inundated woodland areas, each multiple hectares in size. The second population was a pond in Butte La Rose, LA (30°21'22.86"N, 91°43'14.16"W). This freshwater swamp was located near a levee road in the Atchafalaya River basin surrounded by dense forest. The last location was in Sherburne Wildlife Management Area (30°23'29.13"N, 91°39'27.92"W). This ditch was located a half mile after the permit station and was only useable after a very heavy rain.

Recording and Analyzing Calls

At all sites, I located individual males by their calls and captured them in a mesh cage in order to reduce stress induced by handling. Direct handling prevented frogs from calling in the cage, even when encouraged by a chorus recording. I moved the male far enough from the chorus (ca. 10 meters) so that it would not mask the individual male's call during recording. I broadcast a spring peeper chorus recording to entice him to call. Once he was calling, I recorded the vocalization using a Marantz PMD-660 recorder and Sennheiser ME-66 shotgun microphone. Vocalizations were recorded digitally and saved as .WAV files. After recording, I measured the frog's snout-vent length (SVL). All males were toe clipped to prevent redundant sampling. I imported all calls onto a Dell Optiplex GX280 desktop

computer and analyzed them in Sound-Ruler (Gridi-Papp 2007). Recordings from each male consisted of eight to sixteen individual peeps. To avoid pseudoreplication, however, sample sizes were calculated based on individual calling males, not the total number of calls analyzed. Each calling male had one “peep” selected from the middle of the recording to be analyzed for call properties. The parameters that I analyzed were fundamental frequency, dominant frequency at the start (DFS) and end (DFE) of a call, rise time, fall time, pulse duration, and call rate. Only five parameters were needed to construct a synthetic call. These included the dominant frequency at the start of the call, dominant frequency at the end of the call, rise time, fall time, and the call duration. I took the average values recorded from the population and used these parameters for the construction of synthetic calls.

Results

Natural Calls

Calls from 111 individual males (64 from MD and 47 from LA) were analyzed. The mean fundamental frequency was lower in LA frogs compared to MD frogs (Table 1). The MD frogs had an overall higher frequency call including a higher DFS and DFE (Fig. 1). There were significant differences in the DFS (Mann–Whitney U, $p < 0.001$, $N = 111$), DFE (Mann–Whitney U, $p < 0.001$, $N = 111$), and frequency sweep (the difference between the DFE and the DFS) (Mann–Whitney U, $p = 0.003$, $N = 111$) between the two populations. There was no significant difference in size between frogs from LA and MD (27.98 mm and 27.58 mm respectively,

Mann–Whitney U, $p=0.11$, $N=104$). The calls of the spring peeper exhibit a positive frequency sweep, such that the end of the call is a higher frequency than the beginning (Fig. 2). MD frogs covered a wider range of frequencies in their sweep than did LA frogs. The rise and fall times of the calls from both MD and LA were not significantly different (Mann–Whitney U, $p=0.071$ and $p=0.667$, respectively, $N=111$). Call duration and call rate were also not significantly different (Mann–Whitney U, $p=0.124$ and $p=0.407$, respectively, $N=111$), but MD frogs exhibited a slightly faster call rate; LA frogs had a slightly longer call.

Synthetic Calls

Based on average values, I constructed synthetic calls for the MD population and the LA population to be used in phonotaxis experiments. Peeper advertisement calls are simple and nearly all of the energy of the call falls into a single harmonic, which makes only a few parameters necessary for synthesis. To construct a synthetic call, I used five call parameters: DFS (2711 Hz MD, 2535 Hz LA), DFE (3022 Hz MD, 2791 Hz LA), rise time (11.7 ms MD, 10.1 ms LA), fall time (7.7 ms MD, 8.4 ms LA), and pulse duration (155 ms MD, 167 ms LA).

Discussion

I analyzed seven call parameters, but only three were significantly different between MD and LA. These were DFS, DFE, and the frequency sweep throughout the call. Frequency is typically constrained by body size and in frogs is generally

inversely proportional (Doherty & Gerhardt 1984; Sullivan 1992; Gerhardt & Huber 2002). The mean SVL for MD and LA were 27.58 mm and 27.36 mm respectively (Table 1). Since SVL did not differ, size is unlikely to explain the difference in call frequency. I did not measure mass when recording calls so it is possible that there is a size difference between the populations. SVL is normally a good measure of body size (Dashevsky et al. 2013), and I did measure mass of peepers used in another study. For MD frogs, SVL and mass are strongly correlated, indicating that SVL is indeed a good predictor of body size (Fig. 4). If frogs grow allometrically, laryngeal size would scale proportionately to body size. Interestingly, this was not the case for our populations. If strong sexual selection is not present, this effect could be the result of genetic drift.

The frequency sweep was also different between MD and LA frogs. Capranica (1966) suggested that there is a “mating call detector” in the frog brain that is stimulated by the advertisement call at certain frequency peaks. More recently, the match–filter hypothesis suggests that matched filtering can improve the ability of receivers to detect calls of conspecifics in a noisy environment (Gerhardt & Schwartz 2001). The variation in sweep difference could be attributed to the fact that larger sweeps encompass a broader range of frequencies thereby having a better chance of matching the template in the brain of a receiver. This could increase the chances of species recognition and potentially increase locational cues. The detection of these frequencies is completed through the basilar papilla. The basilar papilla detects high frequency sounds (Feng et al. 1975) and it has been suggested in female peepers, that

the basilar papilla may measure total energy and not the specific spectral components of the call (Wilczynski et al. 1984). Males who produce a narrow range of frequencies would have more total energy within their call than a male who produced a wide spectral range. Females might have a preference for a certain frequency, but if the total energy of one call is greater than that of another call with an optimal frequency, the preference for that certain frequency could be washed out. Maryland frogs had a narrower frequency range. Since the sweep contains a narrower range of frequencies, each frequency produced will have a greater intensity and can be transmitted further (Ryan 1992).

The differences found in call properties between populations could potentially be a result of isolation by distance where genetic differentiation increases with geographic distance (Wright 1943; Vortman et al. 2013). These models suggest increasing genetic differentiation among populations and it is not unreasonable to expect similarities in behavioral phenotypes. The two populations I studied are separated by a distance of 1,719 km. In the chowchilla, *Orthonyx spaldingii*, song frequency and structure were variable in distances under 1,000 km (Koetz et al. 2007) and within a few hundred kilometers, bronzed cowbirds were shown to exhibit dialects in their “flight whistle” song (Warren & Nelson 2002). It has also been shown in white-crowned sparrows that a reduction in gene flow is associated with song dialect (MacDougall-Shackleton & MacDougall-Shackleton 2001). However, bioacoustic differences between túngara frog populations (Panama and Costa Rica) are more likely to be predicted by geographic distance than genetic distance (Prohl et

al. 2006). It is still reasonable to expect that a distance of 1,700 km would result in little if any gene flow and explain the differences in the population call parameters (frequency and sweep).

Populations that are separated by water and not land geography still show evidence of variation in calls and songs. Marine mammals that live in colonies and form feeding aggregations show variation in song properties and structure (Bjorgesaeter et al. 2004; Delarue et al. 2009). Differences among these songs have allowed researchers to follow different colonies and track them for conservation assessment. Although anurans do not form these feeding aggregations or colonies, they do exhibit extremely high rates of philopatry (Daugherty & Sheldon 1982; Berven & Grudzien 1990). The discrepancies found between the MD and LA spring peeper populations support the idea that distance can lead to divergence in vocalization phenotypes. If a frog returns to the same ponds every night (and every season), meaning that the home range is relatively small, we can expect that genetic differentiation would be more prevalent than in a species that ranges over relatively large distances.

Latitude can also influence the calls of organisms. Koetz (2007) found that southern chowchillas had lower peak frequencies and narrower bandwidth than northern birds. The spring peepers showed similar results, with the LA (southern) population having lower frequency calls and a narrower sweep (bandwidth) from the start to the end of the call. The chorus frog, *Pseudacris triseriata*, showed clinal variation in dominant frequency with higher frequency values in South Dakota

compared to Kansas populations (Platz 1989). And boreal chorus frogs, *Pseudacris maculate*, in Minnesota had higher dominant frequencies than values found in Platz's study (Bee et al. 2010). The two spring peeper populations are geographically distant and in order to measure the clinal variation in the spring peeper, populations located between MD and LA would be needed to evaluate variation in calls over more intermediate geographic distances.

Chapter 3 (Peeper Mate Choice – Phonotaxis)

Materials and Methods

Phonotaxis

I used a two-choice phonotaxis design to test female mate preferences for male calls. Phonotaxis procedures isolate a female and play different male vocalizations, allowing her to choose what she prefers. This method allows for manipulation of the variables being tested and provides a clear indication of female mate preferences for particular call properties. Since the variables can be manipulated, many questions can be addressed using phonotaxis.

Test Subjects

Protocols for collecting, handling, and testing animals were approved by Salisbury University's Institutional Animal Care and Use Committee (#SU – 0023). I collected females in amplexus from field sites in Louisiana in January, 2014. I placed the pairs in 1 quart plastic bags in a cooler until females were ready to be tested. After trials were complete, females' mass and snout-vent length (SVL) were recorded. Each female received an individual toe-clip identification number and was returned to its capture location (within 2 days).

Behavioral Assays

I conducted two-choice phonotaxis experiments in an arena made from a wooden frame and sound foam attached to the frame to reduce acoustic

reverberations. Ceiling tiles were placed along the outside of the arena and angled along the top to further reduce reverberations. The chamber was visually symmetrical and the arena was illuminated at light levels between 4.08×10^{-10} and 2.1×10^{-11} W/cm². All trials were recorded using an IR converted Logitech C525 HD Webcam and Logitech webcam recording software. Acoustic stimuli were synthetically generated from previously collected call data using Avisoft SASLab Pro (Table 2). The signals were broadcast from Mirage 360° Nanosat satellite loudspeakers and NAD Stereo Integrated Amplifiers (C 316BEE) through Adobe Audition 2.0. The speakers were placed 80 cm apart and equidistant from the center of the chamber where the female was released. I measured and calibrated sound levels at the location of the frog's release using an Extech 407764 sound level meter. Both speakers were calibrated to 86 dB (re. 20 μ Pa, fast, C weighted measure).

At the onset of each trial I placed a female under a funnel at the release point and allowed her a two-minute acclimation period during which auditory stimuli were presented. While under the funnel, females could move freely and change their orientation. After the acclimation period, the funnel was lifted and the female was allowed to choose between the two stimuli. A trial was considered successful when a female entered a choice zone, a 5 cm area around the front and sides of a speaker, and remained within the zone for three seconds. This 3 second rule makes the behavioral assay a more realistic proxy for mate choice. If a female randomly walked in front of a speaker and continued moving, it is unlikely that she would be clasped by a male. Remaining near a male for 3 seconds in nature increases the probability that she

would be clasped by the male. If the female did not make a choice within 10 minutes, failed to move out of the funnel zone within two minutes, ceased movement for two consecutive minutes, or started to climb the walls of the chamber, the trial was excluded.

Experiments

I conducted choice experiments to outline female preferences in the spring peepers of Louisiana. Specifically, I tested for frequency, frequency sweep, duration, and population specific calls (e.g., preference for LA vs. MD call types). The values for high and low parameters of the calls were two standard deviations from the mean, but within the range of recorded calling data (Table 1). If two standard deviations were outside the natural range, then both the high and low values were adjusted to keep them within the range and at equal distances from the mean. Two standard deviations were chosen because preliminary data showed no preference when signals were synthesized one standard deviation from the means. I also tested a synthetic call against a natural call to make sure females did not prefer natural calls over synthetic ones. The synthetic call was an average of all call values and the natural call was matched as closely as possible to those values. If females preferred natural calls, the synthetic calls could not be used to develop preference functions because this indicates that the synthetic call may not be biologically relevant.

Results

Females did not show a preference between natural and synthetic calls, justifying the use of synthetic calls to test for female preferences (binomial test: $p=0.22$, $N=11$). I tested for female preference in the parameters of the calls that exhibited a significant difference between the MD and LA populations (frequency and frequency sweep). I also tested duration of the peep and whether females prefer a local call to a foreign population call. I predicted that females would exhibit preferences similar to documented preferences in other frog species, favoring a lower frequency, a longer duration, and their local call. I hypothesized that a positive sweep and an extreme sweep would be preferred because the sweep could be a means of species recognition. Only one of these predictions was supported.

Females did not show a significant preference for a high versus low frequency call (binomial test: $p=0.15$, $N=15$). I also found no preference for any of the frequency sweep tests. There was no significant difference between an average positive sweep versus negative sweep (binomial test: $p=0.16$, $N=20$) or between an extreme sweep (double the average, 512 Hz) and no sweep (binomial test: $p=0.19$, $N=15$). Females did, however, prefer a long call over a short call (binomial test: $p=0.003$, $N=18$). Lastly, there was no preference for a local call over a foreign call either (binomial test: $p=0.19$, $N=15$).

Discussion

Females did not express a preference for any call parameter except for call duration. In gray treefrogs, *Hyla versicolor*, longer duration calls are indicative of higher male mating success in that females prefer longer calls (Schwartz et al. 2001). Duration was suggested to be an indicator of genetic quality in gray treefrogs as well. Males that could produce longer calls produced offspring with higher survivorship compared to males who made shorter calls (Welch et al. 1998). However, those results could not be reproduced in another study (Doty & Welch 2001) and since I did not rear offspring to test survivorship, there is no evidence that indicates these males are broadcasting genetic quality with the duration of their calls. If a call has a longer duration than another call, the longer call contains a larger sound envelope and has more total energy within the call. This extra energy can generate increased stimulation in the female's auditory system, thereby driving the preference. This is compatible with the sensory drive hypothesis (Ryan & Keddy-Hector 1992; Endler & Basolo 1998). Doherty and Gerhardt (1984) found that female spring peepers preferred a longer call over a shorter call despite the shorter call being at a higher amplitude. The longer call duration excited the female's inner ear more so than a louder, shorter call. The call duration may also serve as another means of species recognition. Strecker's chorus frog, *Pseudacris streckeri*, produces a very similar call to the spring peeper, except the duration of the call is about 20 – 60 ms (Blair & Littlejohn 1960), whereas the spring peeper's duration is approximately 160 ms. The

amount of energy in a 40 ms call may generate call recognition in Strecker's chorus frog, but not a spring peeper.

Despite a lack of preference for different frequencies, the results are still interesting. Females from Louisiana do not prefer low frequency calls as seen in other species (Andersson 1994). Low frequency calls usually indicate larger males. However, I found that even though there was a significant difference in frequency between MD and LA calls, the SVL of the two locations is almost identical. This contradicts a previous study in which the mid-point frequency of the call was negatively dependent on body size (Doherty & Gerhardt 1984). In my sampling, the lower frequency calls broadcast by males are not indicative of a larger male who could potentially fertilize more eggs in a female's clutch. And since the lower calls, in this case, are not a reliable indicator of a larger male, frequency may not be a preferred call parameter. The difference in frequencies could be a byproduct of clinal variation within a species (Platz 1989; Bee et al. 2010). Another study has also shown that spring peepers do not prefer specific frequencies (Schwartz & Gerhardt 1998), except when chorus shaped noise was present (at a much lower amplitude) and the discrepancy between the calls was 900 Hz. Since the signals used in my experiments were only 784 Hz apart (± 2 SD), this could explain why I did not find a preference. Alternatively, Forester and Czarnowsky (1985) found a preference for lower call frequency when their stimuli were only separated by 750 Hz (3500 Hz vs. 2750 Hz). It has also been suggested that the basilar papilla alone is not very good at discriminating between different frequencies of calls (Zakon & Wilczynski 1988),

which would explain why there was no preference for a specific frequency.

Frequency assessment may only become important when females are comparing signals at different amplitudes or intensities (Schwartz & Gerhardt 1998).

These reasons also likely explain why there was no preference for local versus foreign calls. Females did not prefer a Louisiana call over a Maryland call. What makes these calls distinct is primarily difference in frequency. The LA call differed from the MD call by 175 Hz in the DFS, 231 Hz in DFE, and 54 Hz in the sweep. The difference between the two calls is small compared to the experimental stimuli I used to test preferences for different frequencies. When testing for preferences in frequency, the stimuli differed by 784 Hz and when testing for preferences in frequency sweep the difference between the start and the end of the call was 512 Hz. If a preference does not exist between such large differences in the signals, than the smaller differences between the average calls for each population would not be expected to elicit a preference either.

Chapter 4 (Túngara Mate Choice – Multimodal Signal Weighting Phonotaxis)

Materials and Methods

Phonotaxis

I conducted phonotaxis tests, with increasing complexity, to test the role of visual cues in female mate choice. Phonotaxis procedures isolate a female and play her different stimuli and allow her to choose what she prefers. This method allows for the manipulation of the variables being tested and presents clear data when paired with statistical analyses. Since the variables can be manipulated so easily, many questions can be addressed using phonotaxis. These data allow us to build preference functions to visualize a sub-set of a population's preferences for certain traits of a male call.

Samples

I collected amplexed pairs of frogs from two sites at the Smithsonian Tropical Research Institute, Gamboa, Republic of Panama. The first site, Santa Cruz (9°7'13.97"N, 79°42'14.38"W), is a collection of concrete pools and moats used to maintain water flow to cultured plants. The second site, Pre-pipeline (9°7'15.23"N, 79°42'54.53"W), is a small service road near the entrance to Soberania National Park at Pipeline Road. It has many bumps and divots that create small pools where the frogs congregate. Upon collection, I placed pairs in plastic bags and then into a cooler in total darkness for transport. Frogs remained in the cooler for a minimum of

one hour for dark adaption (Taylor et al. 2008). After experiments were completed for the night, I collected morphometric data on each frog and took toe clips for identification and later genetic analysis. All frogs were released at their location of capture at the end of the night.

Behavioral Assays

I conducted two-choice phonotaxis experiments in a semi-anechoic sound chamber (ETS-Lindgren, Austin, TX) with sound foam added to the door and back wall to reduce residual acoustic waves. The chamber was visually symmetrical and light levels were kept consistent at ca. 5.9×10^{-10} (Watts/cm²). All trials were recorded using an Everfocus EHD500IR camera and Ethovision XT 8.5/Media Recorder 2.0. Acoustic stimuli were used from previous experiments (Ryan & Rand 2003). These were natural calls that were shown to vary in their attractiveness relative to one another. I broadcast all acoustic signals from Mirage 360° Nanosat satellite loudspeakers and NAD Stereo Integrated Amplifiers (C 316BEE) through Adobe Audition 2.0. The speakers were 80 cm apart and equidistant from the center of the chamber where the female was released. I measured and calibrated sound levels at the location of the frog's release using an Extech 407764 sound level meter. Both speakers were calibrated to 82 dB (re. 20 μ Pa, fast, C-weighting).

I used a robotic frog as a visual stimulus to weigh the effects of the vocal sac on female choice. I tested whether the presence of the vocal sac paired with an unattractive call would make that call more attractive to females. The robofrogs were

the same used in previous visual experiments (Taylor et al. 2008) and connected to a pneumatic pump that inflated and deflated the vocal sac synchronously with the auditory stimulus. The vocal sac consisted of a urological catheter balloon, painted black with a white vertical stripe down the middle to closely resemble a natural vocal sac. The vocal sac catheter was replaced after every two trials to reduce the probability of rupture during a trial due to overuse (see Taylor et al. 2008 for details).

At the beginning of each trial I placed a female under a funnel at the release point and allowed her a two-minute acclimation period during which auditory stimuli were presented along with the robofrog. While under the funnel, females could move freely and change their orientation. After the acclimation period, the funnel was lifted and the female was allowed to choose between the two stimuli. Trials were considered successful when a female entered and stayed in the choice zone (5 cm diameter area) for three seconds. If the female did not make a choice within 10 minutes, failed to move out of the funnel zone within two minutes, ceased movement for two consecutive minutes, or started to climb the walls of the chamber, the trial was excluded from the data set. A trial was also excluded if a catheter balloon ruptured during the trial.

Experiments

I performed two experiments to test the effects of the vocal sac on female choice. I conducted a control experiment to re-establish female preferences for the calls, previously shown to be differentially attractive (Ryan & Rand 2003). I used a

total of six natural calls and compared their relative attractiveness to one another. These calls were used in previous experiments (Ryan & Rand 2003) and each was deemed to be either an “attractive” or “unattractive” call. I took the known attractive and unattractive calls and pitted them against one another in a phonotaxis only test. Since I was assessing the relative attractiveness of attractive and unattractive calls and not the individual parameters of each call, I pooled the calls into one experiment. There were eight pairs of calls to be tested and four trials per pair, totaling 32 trials. In the second experiment, I placed the robofrog in front of the unattractive call to test if the visual cue increased preference for the unattractive call. This tested the role of multimodal signaling in the mediation of call attractiveness.

Results

In the basic preference test, I hypothesized that females would prefer the attractive calls over the unattractive calls, based on the previous study (Ryan & Rand 2003) that used these calls. In my experimental design, the speakers broadcasting the two calls were placed on the same side of the acoustic chamber instead of on opposite sides as in (Ryan & Rand 2003). Therefore, it was important to confirm that the previously established “attractive” versus “unattractive” calls remained so with this experimental design (Akre et al. 2011; Ryan 2011). Females significantly preferred the attractive calls over the unattractive calls (binomial test: $p < 0.001$, $N = 32$). Twenty-six females chose attractive calls and six females chose the unattractive calls.

In the second multimodal experiment, I placed a robofrog with inflating vocal sac in front of the unattractive call; the other speaker broadcast an attractive call. Based on previous work (Taylor et al. 2008), I predicted that the presence of the inflating vocal sac would increase the attractiveness of the otherwise unattractive call. This was not the case. I obtained identical results to the previous experiment lacking the robofrog; twenty-six different females chose the attractive call and only six choosing the unattractive call paired with the robofrog (binomial test: $p < 0.001$, $N = 32$). The visual stimulus did not modulate the disparity in attractiveness between the calls.

Discussion

Females exhibited a significant preference for attractive calls over unattractive calls in both experiments. The proportion of choices did not change between the experiments either. This indicates that the vocal sac does not enhance the attractiveness of calls when they can distinguish one call from another. Since the vocal sac alone does not elicit a response from females (Taylor et al. 2008), it may not be a sexual signal that serves as a mate attracting ornament. The evolution of the vocal sac has made calling behavior more energetically efficient (Pauly et al. 2006) and therefore probably did not originally evolve for the purpose of communication. The vocal sac then could be described as a cue for other purposes.

When females are presented with the same calls and one is paired with an inflating vocal sac, they significantly prefer the multimodal signal (Taylor et al.

2008). This would indicate that when females are not able to distinguish among calls, they might rely on the inflation of the vocal sac to identify and localize a male. In a dynamic acoustic environment, female túngara frogs will assign calls to sources that provide the smallest relative difference among call parameters. When given a whine with two spatially separated chucks, females will assign the whine with the closest chuck (Farris & Ryan 2011). In a chorus, females typically have more than two males to choose among, making it difficult to distinguish among calling males (Bee & Micheyl 2008). To distinguish among calling males, female gray treefrogs, *Hyla chrysoscelis*, exploit a tactic called dip listening. Here the females are able to catch “acoustic glimpses” of calls when the background or chorus noise decreases momentarily. Female gray treefrogs only needed 6 – 9 pulses, the equivalent of 120 ms, in order to recognize the call as a conspecific and exhibit positive phonotaxis (Vélez & Bee 2011). In the absence of a trill in the túngara male repertoire, the vocal sac could be the equivalent of a less noisy chorus and allow the female to assign a call to a signaler and exhibit phonotaxis.

The next experiment necessary to answer this question is to test what females prefer in a noisy environment. The test would need an attractive call, an unattractive call with the robofrog, and chorus shaped noise to simulate a typical night during the mating season. I predict that the robofrog would enhance the attractiveness of the unattractive call in a noisy environment. In this case, the vocal sac would aid females in assigning calls to specific males. If females still prefer the attractive call in the midst of chorus noise, then the vocal sac may not help females localize calling males

in the middle of a chorus, but simply be a byproduct of calling efficiency. However, it has been shown that when there is temporal asynchrony between the call and the inflation of the vocal sac, females reject that stimulus (Taylor et al. 2011). Females prefer a call by itself over a call that is not in sync with its perspective signaler. These findings suggest a role for the vocal sac in call discrimination and potentially localization.

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Table 1. Mean values for analyzed call parameters for the spring peeper. Means are followed by standard deviations and ranges for their respective location. (MD, N = 64 and LA, N = 47)

Parameters	Maryland	Louisiana
Fundamental Freq.	1455 Hz \pm 11; 1248 – 1734	1381 Hz \pm 13; 1062 – 1593
Start Frequency	2711 Hz \pm 195; 2325 – 3093	2535 Hz \pm 201; 1875 – 2842
End Frequency	3022 Hz \pm 167; 2670 – 3468	2791 Hz \pm 215; 2125 – 2842
Frequency Sweep	311 Hz \pm 193; –187 – 750 Hz	256 Hz \pm 171; 0 – 689 Hz
Rise Time	11.7 ms \pm 5.9; 3 – 31 ms	10.1 ms \pm 6.4; 2 – 34 ms
Fall Time	7.7 ms \pm 6.2; 1 – 45 ms	8.4 ms \pm 8.3; 1 – 55 ms
Call Duration	155 ms \pm 42; 68 – 274 ms	167 ms \pm 46; 77 – 288 ms
Call Rate	62 cpm \pm 27; 29 – 221 cpm	59 cpm \pm 23; 28 – 120 cpm
Snout–Vent Length	27.58 mm \pm 1.91; 24.4 – 30	27.36 mm \pm 1.44; 21 – 30

Table 2. Values for synthetic calls used in spring peeper phonotaxis. All other call parameters of each signal are set at the mean values for the population so the only differences in the calls are the experimental parameters.

Experiment Parameters	Values
Frequency (High/Low)	3031 Hz / 2247 Hz
Sweep (Positive/Negative)	256 Hz / -256 Hz
Sweep (None/Extreme)	0 Hz / 512 Hz
Duration (Short/Long)	74 ms / 258 ms
Maryland/Louisiana (Foreign/Local)	2711 Hz / 2535 Hz

Figure 1. Box plot highlighting the difference in starting frequency (DFS) between Maryland and Louisiana male spring peepers, *Pseudacris crucifer*. On average, Louisiana males call approximately 200 Hz lower than Maryland males. A total of 111 males were sampled between the two locations. Black dots indicate outliers from population medians.

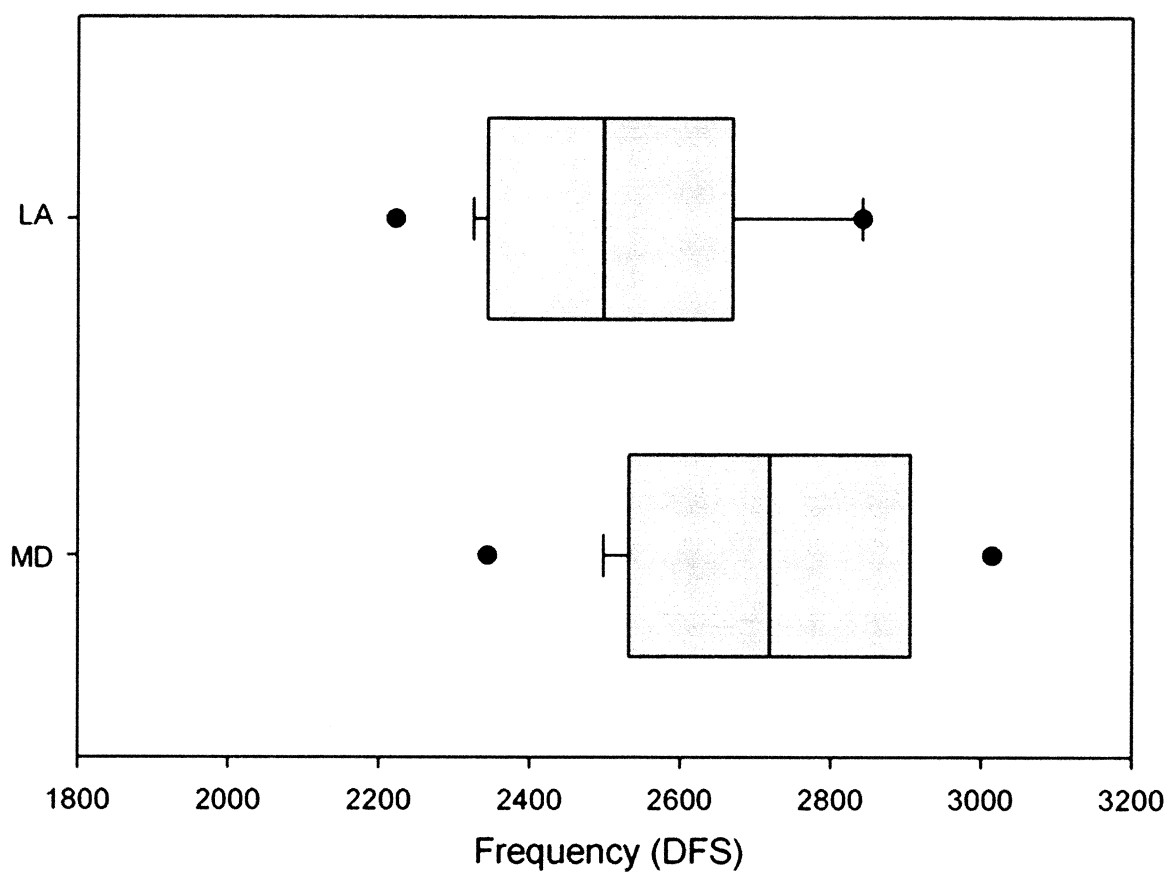


Figure 2. Analysis of spectral and temporal call properties for spring peepers, *Pseudacris crucifer*. Oscillogram (A) of 7 consecutive calls from a single male and isolated peeps (B) highlighting how certain call parameters were measured. The sonogram (C) of a natural call shows the positive frequency sweep and the harmonics. And the power spectrum (D) highlights the relative amplitudes of a single call.

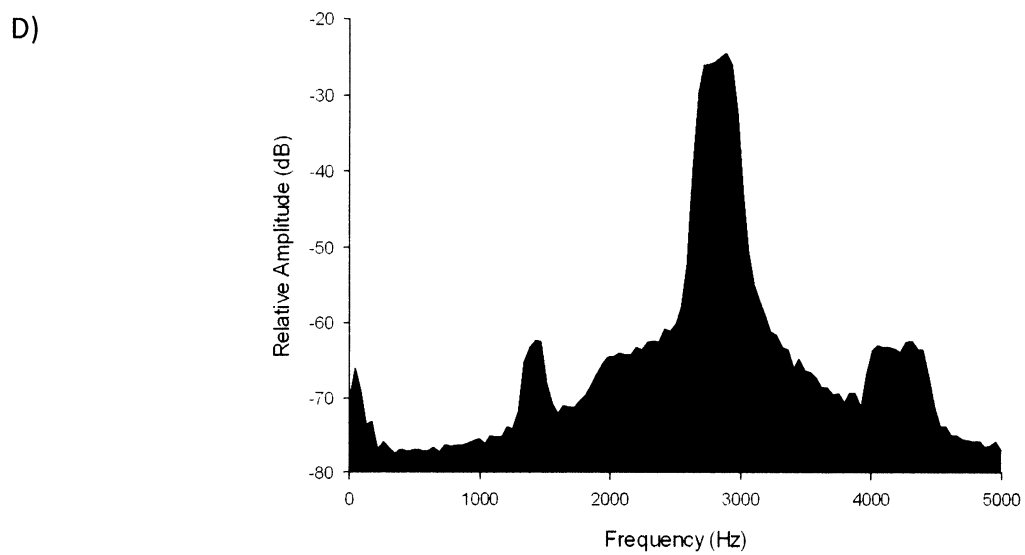
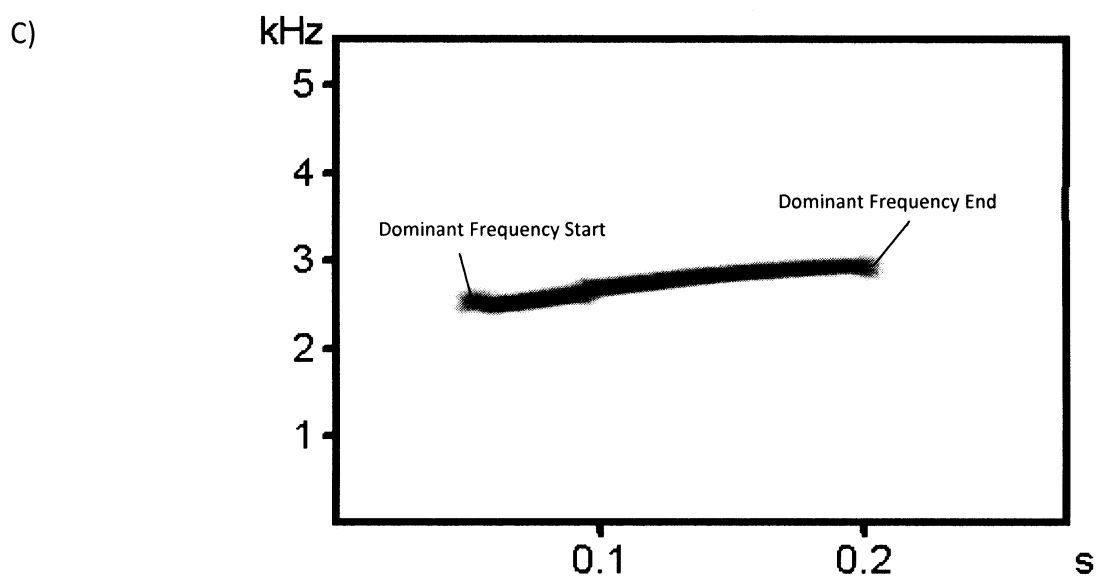
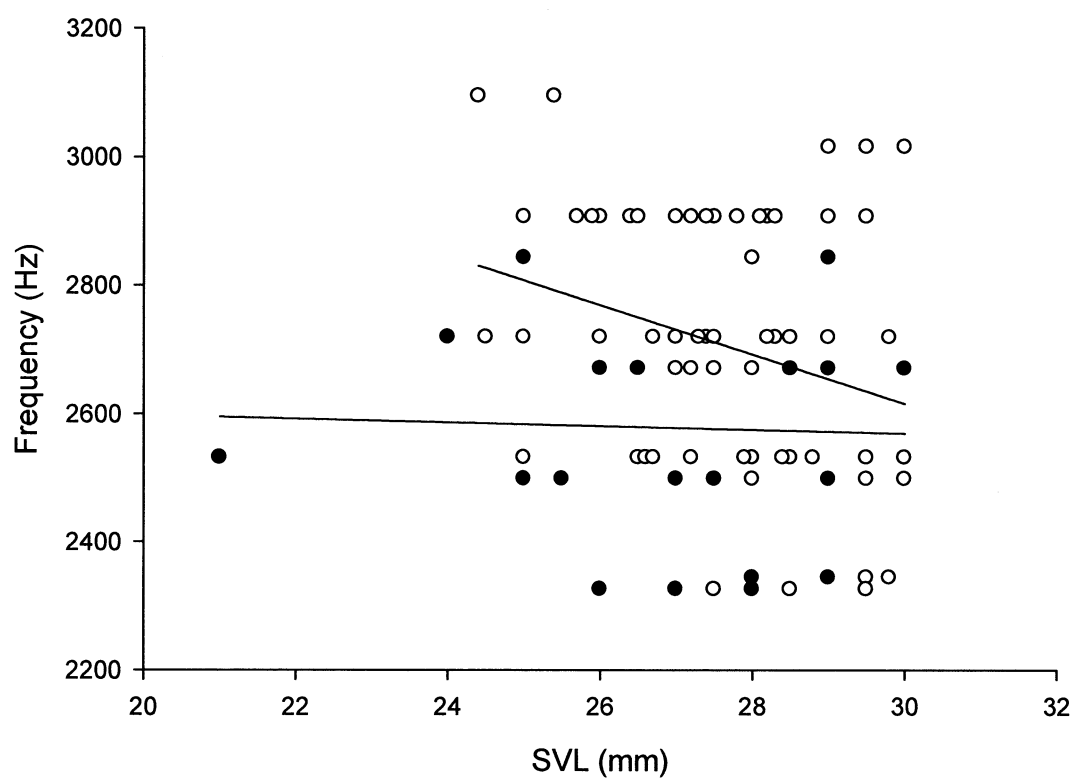


Figure 3. Frequency of a male spring peeper, *Pseudacris crucifer*, call based on his size (SVL). Maryland males are represented by the white circles and Louisiana males are the black circles. There is no correlation between the frequency produced in a call and the SVL of the frog (r^2 for Maryland = 0.078 and $p=0.021$; r^2 for Louisiana = 0.001 and $p=0.824$)



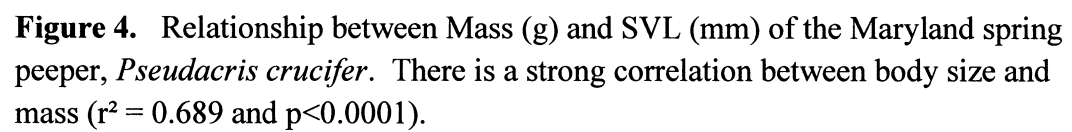
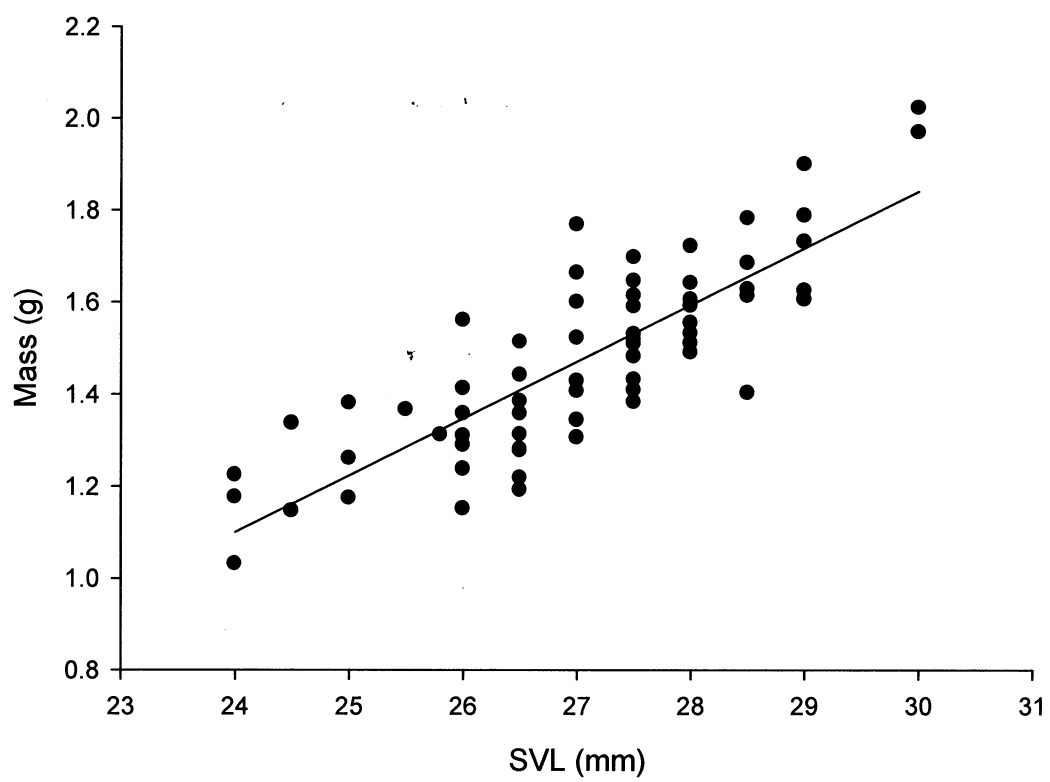


Figure 4. Relationship between Mass (g) and SVL (mm) of the Maryland spring peeper, *Pseudacris crucifer*. There is a strong correlation between body size and mass ($r^2 = 0.689$ and $p < 0.0001$).



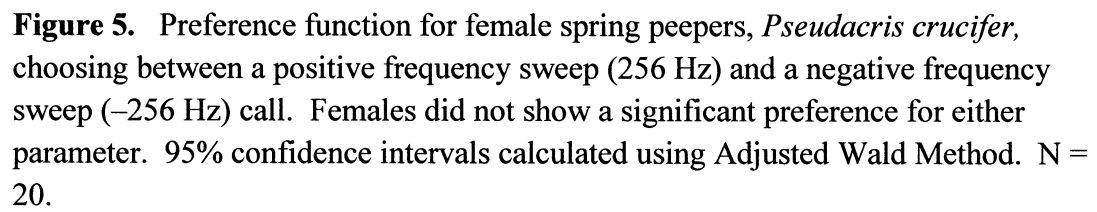


Figure 5. Preference function for female spring peepers, *Pseudacris crucifer*, choosing between a positive frequency sweep (256 Hz) and a negative frequency sweep (–256 Hz) call. Females did not show a significant preference for either parameter. 95% confidence intervals calculated using Adjusted Wald Method. N = 20.

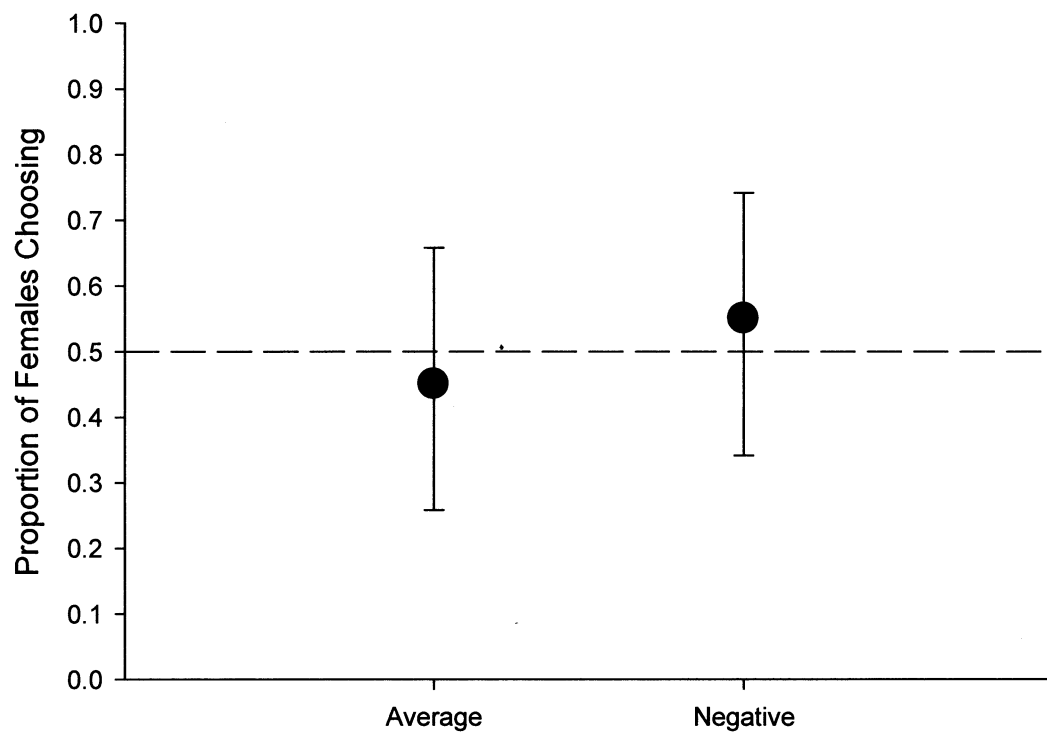


Figure 6. Female spring peeper, *Pseudacris crucifer*, preference function testing between no sweep (0 Hz) and an extended/long frequency sweep (512 Hz). Females did not show a preference for a pure tone (0 Hz) or an extreme sweep. 95% confidence intervals calculated using Adjusted Wald Method. N = 15.

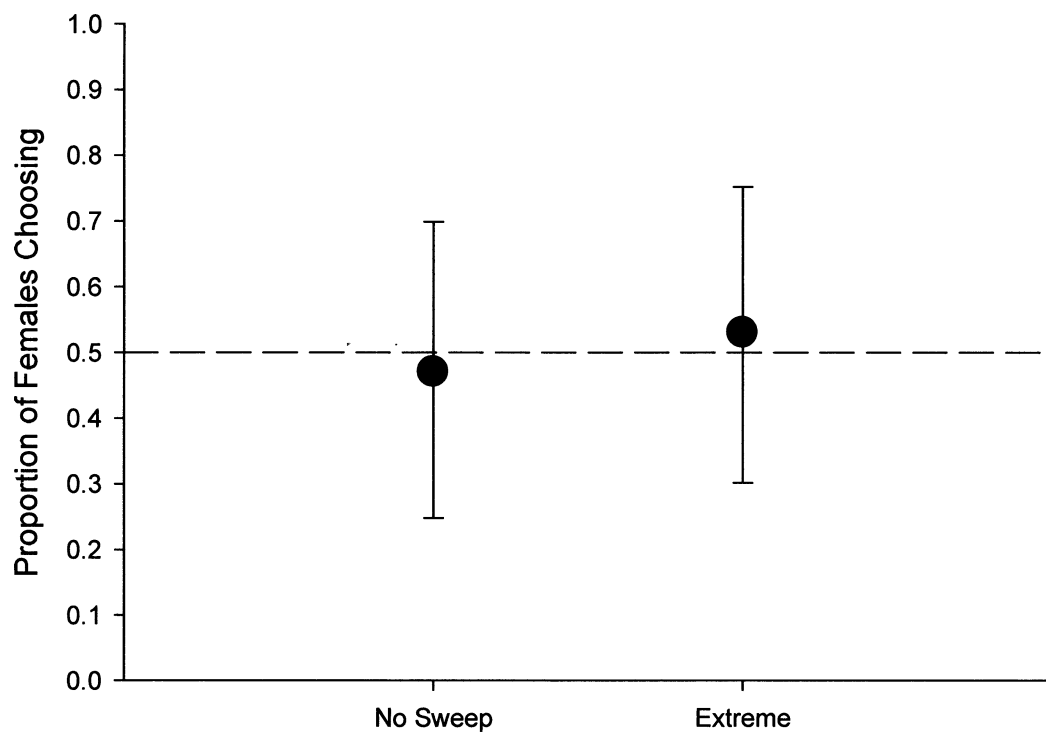


Figure 7. Female spring peeper, *Pseudacris crucifer*, preference function for high (3031 Hz) and low (2247 Hz) frequency calls. Females did not show a preference for low frequency calls as seen in other anurans. 95% confidence intervals calculated using Adjusted Wald Method. N = 15.

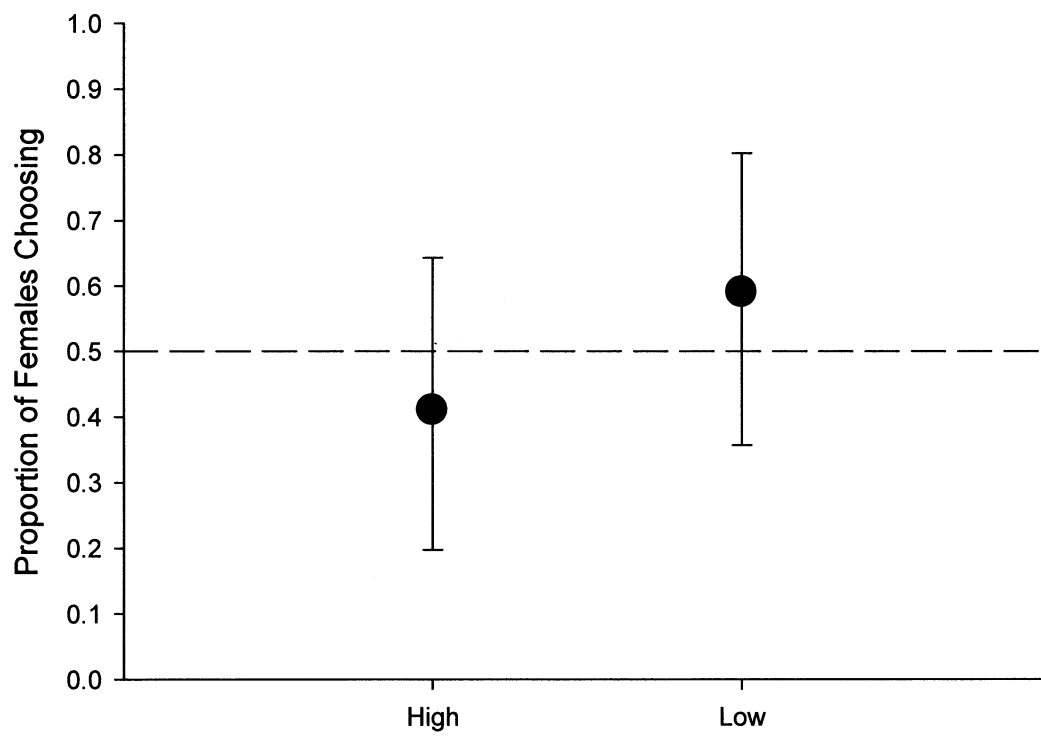


Figure 8. Preference function depicting whether Louisiana female spring peepers, *Pseudacris crucifer*, prefer a call from their local or a foreign population. Call properties mainly differed in frequency. 95% confidence intervals calculated using Adjusted Wald Method. N = 15.

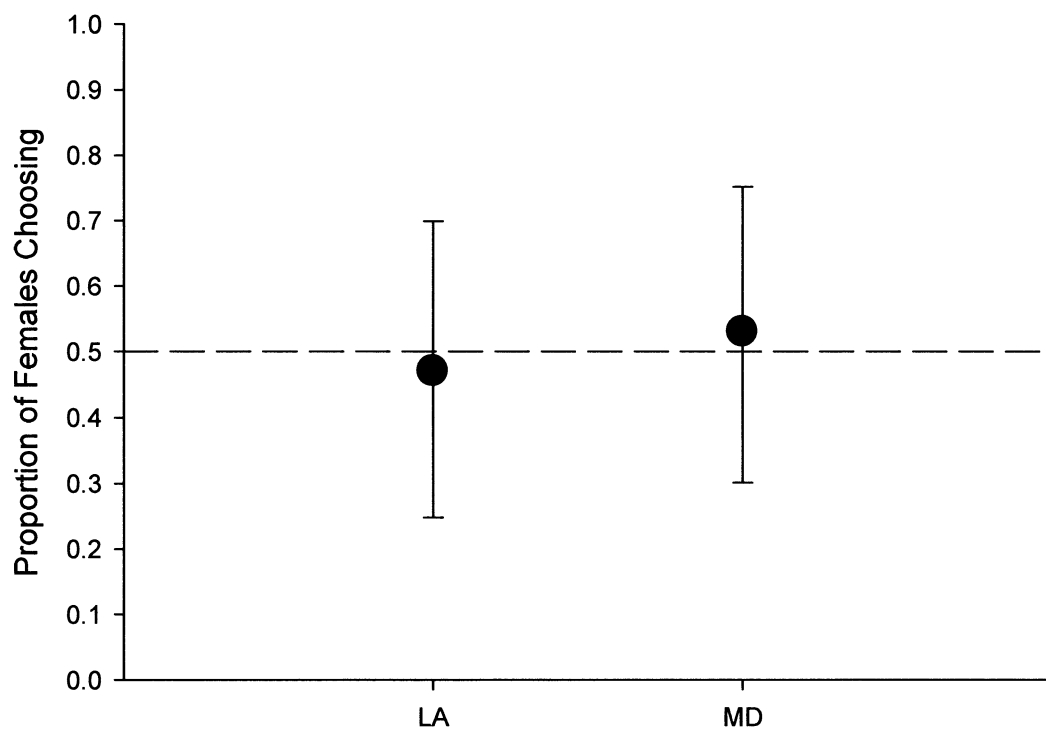


Figure 9. Spring peeper, *Pseudacris crucifer*, preference function for long versus short duration calls. Long calls were 258 ms and short calls were 74 ms. Females showed a significant preference for long calls. 95% confidence intervals calculated using Adjusted Wald Method. N = 18.

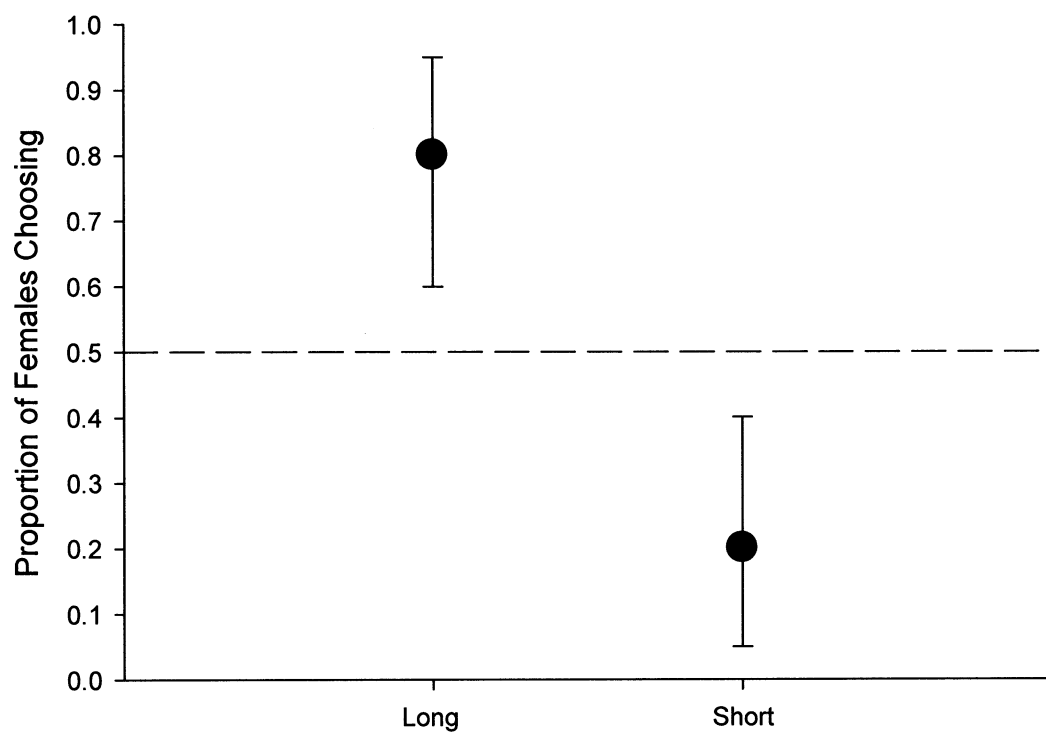


Figure 10. Túngara frog, *Physalaemus pustulosus*, preference function outlining basic preferences for attractive and unattractive calls. Females significantly preferred attractive over unattractive calls. 95% confidence intervals calculated using Adjusted Wald Method. N = 32.

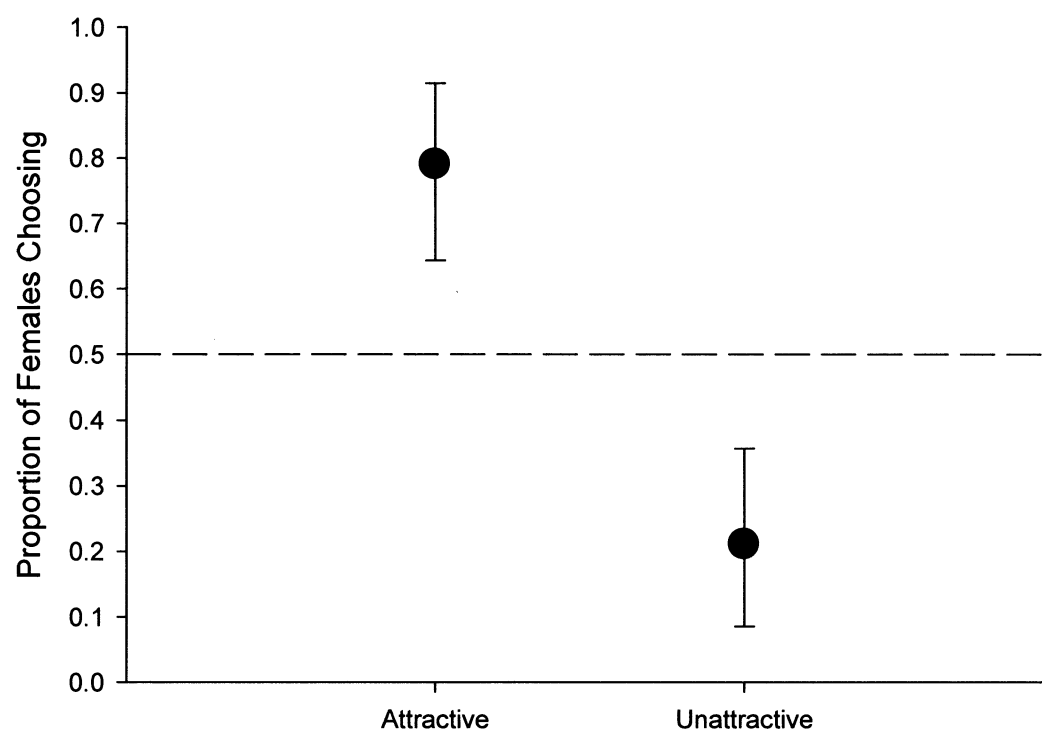


Figure 11. Female túngara frog, *Physalaemus pustulosus*, preference function between attractive calls and unattractive calls paired with a robo-frog. Females still preferred the attractive call over the robotic modulated unattractive call. 95% confidence intervals calculated using Adjusted Wald Method. N = 32.

