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Female preferences for spectral call properties in the western genetic lineage of Cope's gray treefrog (*Hyla chrysoscelis*)

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Abstract

Female frogs discriminate among potential mates based on individual variation in male advertisement calls. While considerable data have accumulated allowing comparisons of female preference functions among species, we still lack fundamental knowledge about how and why the shapes of preference functions for particular call properties vary among populations within all but a few species. Here, we report results from a study aimed at describing female preference functions for spectral call properties in Cope's gray treefrog (*Hyla chrysoscelis*). Widespread throughout the eastern half of North America, Cope's gray treefrog is the diploid member of the cryptic diploid-tetraploid *Hyla versicolor* species complex, and its populations are divided into two distinct genetic lineages (eastern and western). In this study of a western lineage population, we recorded and analyzed the spectral properties of 1000 advertisement calls from 50 males and conducted two-choice phonotaxis experiments to estimate a population-level preference function. Females preferred calls with average frequencies over calls with frequencies that were 2 or 3 semitones (1.4 or 2.1 standard deviations, respectively) lower than the population mean. We observed no behavioral discrimination between calls with average and higher-than-average frequencies. Preferences discriminating against low-frequency calls were weak and were abolished by attenuating the preferred average call by 3 dB. We discuss these results in light of previous studies of eastern lineage populations, geographic variation in female preference functions, and the potential adaptive value of discriminating against calls with low frequencies.

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Ethical Standards

This study complies with the current laws of the U.S.A. and was approved by the University of Minnesota Institutional Animal Care and Use Committee (0809A46721).

Keywords

character displacement; communication; geographic variation; mate choice; preference function; spectral properties

Introduction

Communication signals facilitate mate recognition and can elicit robust mate preferences, usually in females (Ryan and Keddy-Hector 1992; Ryan and Rand 1993; Andersson 1994). Preference functions describe how female preferences vary as a function of male trait values and are useful for inferring patterns of past or current selection on male signals (Gerhardt 1991; Ryan and Rand 1993; Ritchie 1996; Wagner 1998; Murphy and Gerhardt 2000). For example, stabilizing preferences occur when one trait value (e.g., the species average) is preferred over both higher and lower trait values; stabilizing preferences are commonly observed for traits that function in mate recognition (Gerhardt 1991). Females have directional preferences when they prefer traits with relatively higher values (or conversely, with relatively lower values) over other trait values; directional preferences can select for exaggerated traits (Ryan and Keddy-Hector 1992). Threshold preferences can occur when females reject males with traits below a certain value while exhibiting little or no preference for males with above-threshold trait values (Schwartz et al. 2001). Importantly, female preferences are by no means static and can vary substantially within and between species (Gerhardt 1994; Ritchie 1996; Simmons et al. 2001; Boughman et al. 2005; Grace and Shaw 2011).

Investigating population-level variation in preference functions provides insight into factors structuring phenotypic diversity within species and behavioral mechanisms driving or maintaining reproductive isolation between groups (Ritchie 1996; Jennions and Petrie 1997; Foster and Endler 1999; Ptacek 2000; Coyne and Orr 2004). Studies of anurans are particularly informative in this regard because females readily respond to synthetic calls that can be easily manipulated to study the role of signals in mate recognition and mate preferences (reviews in Ryan 2001; Gerhardt and Huber 2002). Spectral properties of male advertisement calls feature prominently in female mate choice in frogs (reviewed in Gerhardt and Huber 2002). Due to biophysical constraints of sound production (Martin 1972; Gerhardt and Huber 2002), the spectral properties of frog calls exhibit little variation within individuals (Gerhardt 1991), and they tend to be negatively correlated with male body size both within a species (e.g., Castellano and Giacoma 1998; Howard and Young 1998) and among species (e.g., Castellano et al. 2002; Hoskin et al. 2009). Thus, call frequency reliably predicts individual differences in body size among conspecific males and could function as a potential cue for discriminating among some conspecific and heterospecific males.

Across anuran species, there is considerable variation in the shapes of female preference functions for spectral call properties (Gerhardt and Huber 2002). In several species, for example, females show directional preferences for calls with lower frequencies (e.g., Ryan 1983; Dyson and Passmore 1988; Morris and Yoon 1989; Ryan et al. 1992; Márquez 1995; Márquez and Bosch 1997; Wollerman 1998). Female preferences for lower-frequency calls provide a mechanistic explanation for large-male mating advantages reported in some frogs (Morris 1989; Morris and Yoon 1989; Morris 1991; Wollerman 1998). In other species, however, females exhibit stabilizing or only weakly directional preferences for spectral properties (e.g., Castellano and Giacoma 1998; Murphy and Gerhardt 2000; Tarano and Herrera 2003; Gerhardt 2005; Poole and Murphy 2007). In still others, females exhibit no frequency-based preferences when choosing among alternatives falling within the natural

range of variation (e.g., Rosso et al. 2006). In light of these species differences, it is impossible (and inappropriate) to make generalizations about the shape of female preference functions for spectral call properties in frogs. Moreover, preferences for spectral properties can be evolutionarily labile within a species (Ryan et al. 1992; Márquez and Bosch 1997; Höbel and Gerhardt 2003), making it difficult to generalize about preference functions for single species without data from multiple populations.

Our aim in this study was to investigate the relationships between spectral properties of male calls, male morphological phenotype, and female preferences in a population of Cope's gray treefrog (*Hyla chrysoscelis*). Common throughout the eastern United States, Cope's gray treefrog is the diploid member of a cryptic diploid-tetraploid species complex; the eastern gray treefrog (*Hyla versicolor*) is the tetraploid (Ralin 1977; Ptacek et al. 1994; Holloway et al. 2006). Genetic analyses of mitochondrial DNA (mtDNA) have shown that populations of *H. chrysoscelis* can be sub-divided into an eastern and a western lineage, with 2.3% sequence divergence between lineages in the *cytochrome b* gene (Ptacek et al. 1994). The geographic distributions of both genetic lineages of *H. chrysoscelis* are partially sympatric with that of *H. versicolor*. Males of both species produce pulsatile advertisement calls with bimodal frequency spectra, with a fundamental frequency between about 1.0 kHz and 1.4 kHz and a dominant second harmonic between about 2.0 kHz and 2.8 kHz (Fig. 2a; Gerhardt 2001). Previous studies of eastern lineage populations of *H. chrysoscelis* have shown directional female preferences for lower-than-average frequencies in Indiana (Morris and Yoon 1989) and higher-than-average frequencies in Missouri (Gerhardt 2005). To date, however, we know nothing about how these preference functions compare with those in western lineage populations. Here, we characterize the relationships between spectral properties and male size and condition, and estimate the population-level female preference function for call frequency in a western lineage population in Minnesota. We discuss our findings with reference to potential selective explanations for intraspecific divergence in female preferences in this species.

Methods

Subjects

This study was conducted between May and July of 2006, 2009, and 2011 using Cope's gray treefrogs (*H. chrysoscelis*) sampled from four adjacent counties in east-central Minnesota. Ptacek et al. (1994) determined that individuals from this area of Minnesota belong to the western mtDNA lineage. Acoustic recordings were made in the Carver Park Reserve (Carver County; 44°52'49.08" N, 93°43'03.11" W), the Lake Maria State Park (Wright County; 45°19'11.81" N, 93°57'07.74" W), the Tamarack Nature Center (Ramsey County; 45°06'08.50" N, 93°02'28.89" W), and the Crow Hassan Park Reserve (Hennepin County; 45°11'18.26" N, 93°38'27.80" W). Behavioral tests were conducted with females collected in the Carver Park Reserve and the Lake Maria State Park. The average pairwise distance between our field sites was 49 km (range: 27 km to 75 km). Populations of *H. chrysoscelis* in the Carver Park Reserve occur in close allopatry (< 10 km) with the eastern gray treefrog *H. versicolor*; the two species breed syntopically at our three other field sites.

Natural variation in spectral properties

Acoustic recordings—In 2006 and 2009, we recorded 20 consecutive calls from each of 50 males (1000 calls total) during peaks of seasonal and nightly vocal activity. Digital recordings (44.1 kHz sampling rate, 16-bit resolution) were made using a Marantz PMD 670 solid-state digital recorder (D&M Professional, Itasca, IL, USA) and a handheld Sennheiser ME66 microphone (Sennheiser USA, Old Lyme, CT, USA) mounted on a MZS 20-1 suspension grip inside a Sennheiser MZW 60-1 blimp windscreen. The tip of the

microphone was positioned approximately 1 m from the focal animal. Males at our study sites usually call on or near the surface of the water from vegetation that is partially submerged; therefore, males were usually in direct contact with the water while calling. Immediately after each acoustic recording, we used a quick-reading cloacal thermometer (Avinet Inc., Dryden, NY, USA) to record to the nearest 0.1°C the water temperature and the wet-bulb air temperature at the male's calling site. Water and wet-bulb air temperatures ranged between 15.0 and 29.0 °C ($\bar{X} \pm \text{SD} = 22.5 \pm 3.1$ °C; $N = 50$) and 10.8 and 24.0 ($\bar{X} \pm \text{SD} = 17.3 \pm 3.6$ °C; $N = 50$), respectively. For a subset of recorded males ($N = 44$), we also captured the individual and measured its snout-vent length (SVL) to the nearest 0.1 mm using dial calipers and its body mass to the nearest 0.1 g using Pesola spring scales. Snout-vent lengths and masses ranged between 33.6 mm and 43.5 mm ($\bar{X} \pm \text{SD} = 38.8 \pm 2.4$ mm; $N = 44$) and 2.8 g and 5.9 g ($\bar{X} \pm \text{SD} = 4.3 \pm 0.9$ g; $N = 44$), respectively. Data for SVL and mass were used to compute an index of body condition (length-independent mass) by dividing the residuals from a linear regression of the cube root of mass on SVL by SVL (Baker 1992). We released males at their calling site immediately following measurements of temperature, SVL and mass. To avoid marking individual males (e.g. by toe-clipping), we assured that no male was recorded more than once by making our recordings in widely separated areas both within and between nights at any one particular field site.

Acoustical and statistical analyses—We used Raven v1.2.1 (Cornell Lab of Ornithology, Ithaca, NY, USA) to measure the two prominent spectral peaks composing the bimodal frequency spectrum of advertisement calls (Fig. 1a). These two peaks correspond to the first and second harmonics, and they were measured from average power spectra computed over the duration of each call from the beginning of the first pulse to the end of the last pulse (FFT size = 1024 points, Hanning window, 75% overlap, 0.043 kHz resolution). We also measured the relative amplitude of the two spectral peaks (in dB). Preliminary analysis indicated that fundamental frequency, dominant frequency and the relative amplitudes of the two spectral peaks varied according to temperature (fundamental frequency: Pearson $r = 0.46$, $p < 0.01$; dominant frequency: $r = 0.28$, $p = 0.05$; relative amplitude: $r = 0.40$, $p < 0.01$). Therefore, the frequencies of both spectral peaks and the relative amplitude were separately adjusted for each call of each male to a common temperature of 20 °C following Platz and Forester (1988). We selected the most appropriate temperature (water or wet-bulb in air) for each male based on his calling position at the time of recording. Average fundamental and dominant frequencies were calculated for each male prior to further statistical analysis. We used coefficients of variation ($\text{CV} = \text{SD} / \bar{X} \times 100\%$) to quantify within-individual (CV_W) and among-individual (CV_A) variability in spectral properties (Gerhardt 1991). We calculated CV_W based on the variation observed over 20 consecutive calls for each male. We calculated CV_A based on the grand mean and standard deviation computed over all 50 individual means.

We used separate multiple linear regressions to assess the effects of male size on mean fundamental and dominant frequencies using SVL and mass as the predictor variables ($N = 44$). Because body condition was derived from SVL and mass, we fit separate models assessing the relationship between body condition and mean values of each call frequency ($N = 44$). All trait values were standardized to a mean of 0.0 and a standard deviation of 1.0 prior to analysis. All of the regression models satisfied assumptions of linearity and the residuals were normally distributed (Kolmogorov Smirnov test: $p > 0.05$ for all models). We did not detect significant multicollinearity between independent factors where multiple regression was used (variance inflation factor for both models = 3.96). To facilitate more direct comparisons with previous studies, we also report the Pearson product-moment correlations between each frequency and SVL, mass, and body condition ($N = 44$).

Female preferences for spectral properties

General testing protocol—Using phonotaxis as a behavioral assay, we conducted two-alternative choice experiments (Gerhardt 1995) with gravid females ($N = 40$) collected in amplexus with males between 2200 h and 0200 h. Amplexed pairs were returned to the lab and kept at 2 °C until testing to prevent egg deposition. At least 30 min prior to testing, pairs were placed in an incubator to allow their body temperatures to reach 20 (± 1)°C. Females were separated from their male partner during a test and then returned to them in the incubator between trials. Frogs collected for behavioral experiments were returned to their location of capture within three days.

Phonotaxis trials were conducted in a 2 m circular test arena located in a temperature-controlled ($20 \pm 1^\circ\text{C}$), hemi-anechoic chamber (Industrial Acoustics Co., Bronx, NY; inside dimensions: 300 cm $\times$ 280 cm $\times$ 216 cm, L $\times$ W $\times$ H). The arena walls were 60 cm high and made from hardware cloth covered in visually opaque but acoustically transparent black cloth. Stimuli were delivered from a Dell Optiplex 980 computer (Dell Inc., Round Rock, TX, USA) using Adobe Audition 1.5 (Adobe Systems Inc., San Jose, CA, USA) interfaced with an M-Audio FireWire 410 multichannel soundcard (M-Audio, Irwindale, CA, USA). The two alternatives in a test were amplified (HTD DMA-1275; Home Theater Direct, Inc., Plano, TX, USA) and delivered to separate speakers (Orb Audio Mod1; Orb Audio LLC, New York, NY, USA) located on the floor of the sound chamber 2 m and 180° apart, just outside the arena wall and facing toward the arena's center.

At the beginning of each trial, a single female was placed in the center of the arena in an acoustically-transparent release cage. We allowed the female to acclimate in the release cage for 1 min before we began broadcasts of two temporally alternating test stimuli. After four repetitions of the two stimuli, the female was released remotely from outside the chamber using a rope and pulley system. Tests were conducted under infrared (IR) illumination, recorded using an IR-sensitive camera mounted above the arena from the chamber ceiling, and scored in real time by two observers who viewed the arena on a monitor located outside the chamber. A choice was defined as the approach of a female to within 10 cm of a speaker. All sound amplitudes were calibrated at distances of 1 m in dB SPL (re 20 μPa , fast RMS, C-weighted) by positioning the microphone of a Larson Davis System 834 sound level meter (Larson Davis, Depew, NY, USA) at the center of the arena at the approximate location of a subject's head.

Acoustic stimuli—In twelve separate two-alternative choice tests, females were required to choose between one of two digitally synthesized calls (20 kHz sampling rate, 16 bit resolution) with different spectral properties but identical temporal properties (Fig. 1b). Each stimulus call was created using custom scripts in MATLAB vR2010b (Mathworks, Natick, MA, USA) and was composed of a series of 30 pulses having temporal properties close to the average values of calls recorded in our study populations after adjusting to 20°C (10-ms duration, 3.5-ms onset, 6.5-ms offset, 10-ms interpulse interval, 50% duty cycle, 50 pulses/s). The amplitude envelope of each call was shaped with a 50-ms linear onset. Each pulse was constructed by adding two harmonically related, phase-locked sine waves with frequencies corresponding to the fundamental frequency (first harmonic) and dominant frequency (second harmonic) in the bimodal spectrum. The amplitude of the fundamental frequency (relative to the dominant frequency) was adjusted for each stimulus such that the average during playback was -8 dB ($\pm 1 \text{ dB}$ range) across the frequencies tested in our study. During a test, each call repeated with a 5 s period, and the two calls alternated in time such that each call was preceded and followed by silent intervals of equivalent duration.

We constructed an average call with a dominant frequency of 2.50 kHz and a corresponding fundamental frequency of 1.25 kHz; these values are close to the population mean (see

Results). We created six alternatives to this average call in which both spectral peaks had frequencies that were 1, 2, or 3 semitones (ST) above or below the corresponding frequencies of the average call (Fig. 1b). Commonly used in psychophysical studies of hearing, semitones represent a measurement of frequency difference based on a constant ratio (1 ST equals 1/12 of an octave) and they were used in this study for purposes of consistency with previous and ongoing studies of hearing in gray treefrogs (Nityananda and Bee 2011; Schrode and Bee 2012). Given the measured standard deviation ($SD = 0.20$ kHz) of dominant frequency reported below, frequency differences of ± 1 ST, ± 2 ST, and ± 3 ST corresponded to differences of about ± 0.7 SD, ± 1.4 SD, and ± 2.1 SD, respectively. We chose a maximum difference of ± 3 ST so that all tested values of frequency in our stimulus calls remained within the natural range of variation.

Preference functions—We conducted an initial experiment in which females ($N = 20$) were tested in six two-choice tests pairing the average call against alternatives differing in frequency by ± 1 ST, ± 2 ST, or ± 3 ST. In these tests, both alternatives were broadcast at 85 dB SPL, a sound pressure level within the range reported for this species in both Missouri (Gerhardt 1975) and Minnesota (Love and Bee 2010). We found that females showed strong discrimination against the two calls with the lowest frequencies (-2 ST and -3 ST) in favor of the average call. In a follow-up experiment, we assessed the strength of these preferences by repeating these two tests (average *versus* -2 ST and average *versus* -3 ST) with a second group of females ($N = 20$) after attenuating the preferred average call by 3 dB, 6 dB, or 9 dB relative to the lower-frequency alternatives presented at 85 dB SPL. Attenuation of a call decreases its attractiveness (Bee et al. 2012); thus the amount of attenuation required to elicit equalization or reversal of preference is an indication of preference strength (Gerhardt et al. 2000). In both experiments, the test order was randomized for each subject, and both the speaker broadcasting each alternative call and the call initiating the alternating sequence were counterbalanced across subjects. We tested the null hypothesis that equal proportions of females (0.50) would approach each of the two alternatives using two-tailed exact binomial tests. We also report 95% binomial proportion exact confidence intervals (CI) and effect sizes g (Cohen 1988).

Results

Natural variation in spectral properties

The average fundamental frequency ranged between 1.11 kHz and 1.53 kHz ($\bar{X} \pm SD$ [in kHz and ST] = 1.31 ± 0.09 kHz, -1.23 ST, $+1.15$ ST; $N = 50$; Fig. 2a). Average dominant frequency ranged between 2.08 kHz and 2.91 kHz ($\bar{X} \pm SD$ = 2.51 ± 0.20 kHz, -1.44 ST, $+1.33$ ST; $N = 50$; Fig. 2b). The average amplitude of the fundamental frequency relative to the dominant frequency ranged between -4 dB and -23 dB ($\bar{X} \pm SD$ = -11 ± 3 dB; $N = 50$). The CV_A values for fundamental frequency and dominant frequency were 6.9% and 8.0%, respectively. The CV_A value for relative amplitude was 29.3%. These estimates of CV_A were uniformly higher than our estimates of mean CV_W for fundamental frequency ($\bar{X} = 1.3\%$; range = 0% to 5.2%), dominant frequency ($\bar{X} = 0.9\%$; range = 0% to 3.4%) and relative amplitude ($\bar{X} = 6.4\%$; range = 1.4% to 20.5%).

Call frequencies were significantly related to male body size (fundamental frequency: adjusted $R^2 = 0.13$, $F_{2,41} = 4.03$, $p = 0.02$; dominant frequency: adjusted $R^2 = 0.26$, $F_{2,41} = 8.70$, $p < 0.01$). Specifically, both fundamental frequency ($\beta = -0.60$, $p < 0.05$, Fig. 2c) and dominant frequency ($\beta = -0.75$, $p < 0.01$, Fig. 2d) were significantly negatively related to mass; neither was significantly related to SVL (fundamental frequency: $\beta = 0.23$, $p = 0.42$, Fig. 2e; dominant frequency: $\beta = 0.26$, $p = 0.33$, Fig. 2f). Separate regressions of call frequencies on body condition indicated that dominant frequency was negatively related to

condition (fundamental frequency: adjusted $R^2 = 0.05$, $\beta = -0.27$, $F_{1,42} = 3.27$, $p = 0.08$, Fig. 2g; dominant frequency: adjusted $R^2 = 0.10$, $\beta = -0.35$, $F_{1,42} = 5.79$, $p = 0.02$, Fig. 2h).

Female preferences for spectral properties

When both stimuli were broadcast with equal amplitudes (85 dB SPL), the proportions of females choosing the alternative to the average call did not differ significantly from 0.5 for frequency differences of -1 ST, $+1$ ST, $+2$ ST, and $+3$ ST (Fig. 3; Table 1). However, the proportion of females choosing the average call was significantly greater than 0.5 when the frequency of the alternative was -2 ST and -3 ST (Fig. 3; Table 1). These results indicated that females preferred calls with average frequencies over alternatives with frequencies that were 2 to 3 semitones below average. We found no evidence that females preferred calls with higher-than-average over average frequencies (Fig. 3; Table 1). Discrimination against calls with lower-than-average frequencies in favor of average calls was weak and was abolished when the sound level of the average call was attenuated by 3 dB or more (Fig. 3; Table 1). The proportions of females selecting alternatives to average calls did not differ from 0.5 when the frequency difference was -2 ST and the average call was attenuated by 3 dB, 6 dB, or 9 dB, nor when the frequency difference was -3 ST and the average call was attenuated by 3 dB or 6 dB. Significantly more than half of females chose the -3 ST alternative when the average call was attenuated by 9 dB (Fig. 3; Table 1).

Discussion

Natural variation in spectral properties

Males of the western mtDNA lineage of Cope's gray treefrog in Minnesota produced calls with a bimodal spectrum having frequency peaks near 1.31 kHz and 2.51 kHz. Gerhardt (1974) reported similar mean (± 2 SE) values of dominant frequency for western lineage populations in Texas (2.57 ± 0.07 kHz) and eastern lineage populations in Georgia and South Carolina (2.45 ± 0.05 kHz). In their study of eastern lineage populations from Indiana, Morris and Yoon (1989) reported a mean ($\pm$ SD) fundamental frequency of 1.35 ± 0.1 kHz. More recently, Gerhardt (2005) reported mean values of 1.25 kHz and 2.39 kHz for fundamental and dominant frequency, respectively, in eastern lineage populations from Missouri. Together, these studies indicate a high degree of similarity in spectral call properties across a wide geographic range and across both identifiable lineages.

The frequencies of the two peaks exhibited little variation within individuals, with mean within-male CVs less than 1.4%, compared to variation exhibited among individuals (≈ 7 –8%). This finding is consistent with numerous studies of frogs showing that spectral properties tend to be “static” features of frog calls (Gerhardt 1991) that exhibit little variation within individuals (e.g., Bee and Gerhardt 2001; Bee et al. 2001, 2010; Castellano et al. 2002; Friedl and Klump 2002; Bee 2004; Gasser et al. 2009). Most frogs possess limited ability (e.g., compared with songbirds) to modulate spectral properties of their calls and the biophysics of sound production constrains the frequencies males can produce (Gerhardt and Huber 2002). These constraints, coupled with individual size differences, give rise to greater among-individual than within-individual variation in spectral properties. Previous studies and our study indicate that females of *H. chrysoscelis* could potentially use spectral call properties to assess male body size. Both frequency peaks were inversely related to male mass in the present study, though these relationships were not particularly strong; we found no relationship between body length (SVL) and frequency after accounting for the effects of body mass (Fig. 2). Gerhardt (2005) reported that fundamental frequency ($r = -0.63$) and dominant frequency ($r = -0.79$) were negatively correlated with body length. Morris and Yoon (1989) also reported a negative relationship ($r = -0.66$) between fundamental frequency and body length. Neither Morris and Yoon (1989) nor Gerhardt

(2005) reported the relationships between call frequency and body mass or condition. Our study extends this earlier work by demonstrating that dominant frequency could also be used to predict a male's condition (Fig. 2).

Female preferences for spectral properties

At the population level, female *H. chrysoscelis* of the western mtDNA lineage in Minnesota exhibited a relatively weak threshold preference function for calls with close to average (-1 ST or -0.7 SD) or higher frequencies within the range of natural variation. Females discriminated against calls with lower-than-average frequencies (i.e., -2 ST or -1.4 SD or more below average) in favor of calls with average frequencies, but they did not behaviorally discriminate between calls with average and higher-than-average frequencies (Figs 3 & 4a). Preferences for calls with an average frequency over those with lower-than-average frequencies were weak and were abolished by attenuating the average call by just 3 dB (Fig. 3). The preference function described here differs from those reported in previous studies of

Cope's gray treefrog, an important finding that we highlight in Figure 4. In their study of populations of the eastern mtDNA lineage in Indiana, Morris and Yoon (1989) conducted laboratory tests showing that females preferred calls with lower-than-average frequencies (-1 SD) over higher-than-average frequencies ($+1$ SD) (Fig. 4b). Morris and Yoon (1989) also reported that females preferred calls with lower-than-average frequencies (-1 SD) over average frequencies in a 2.5:1 ratio (Fig. 4b), though this comparison was not statistically significant at the small samples sizes tested ($N=7$). The preference for low-frequency (-1 SD) calls over high-frequency ($+1$ SD) calls was abolished in additional tests when the preferred low-frequency call was attenuated by 6 dB relative to the high-frequency alternative; smaller amplitude differences were not tested. This same preference was reversed when signal amplitudes were equivalent between the two alternatives, but the high-frequency alternative had a higher call rate (Morris and Yoon 1989). Together, the results of Morris and Yoon (1989) suggest females in their population had weak directional preferences for the lower-frequency calls typical of larger males. More recently, Gerhardt (2005) reported that females from eastern lineage populations of *H. chrysoscelis* in Missouri preferred calls with above-average frequencies ($+1.25$ SD) over calls with average frequencies (Fig. 4c). However, this preference was far from unanimous ($\approx 2.6:1$, $N=29$), and all other two-alternative choice tests conducted with calls having frequencies in the natural range of variation failed to reveal significant preferences (Fig. 4c). Gerhardt (2005) concluded that females were weakly biased toward higher-frequency calls in his study populations. In the same study, Gerhardt (2005) showed that females of the northwestern lineage of the eastern gray treefrog, *H. versicolor*, exhibited stabilizing preference functions (Fig. 4d), preferring calls with average frequencies over those with both lower and higher frequencies (see also Gerhardt and Brooks 2009). However, it is worth noting that female *H. versicolor* only discriminated against higher-than-average frequencies in favor of an average call when the frequency difference was $+3$ SD (Fig. 4d), a difference placing the high-frequency alternative nearly outside the range of natural variation (see Fig. 2 in Gerhardt 2005). Across the range of frequency differences between -0.5 SD and $+2$ SD, females had no significant preferences (Fig. 4d; Gerhardt 2005), a result similar to the lack of preferences we found in *H. chrysoscelis* over a similar range of frequency differences (-0.7 SD to $+2.1$ SD). Taken together, our study and those by Morris and Yoon (1989) and Gerhardt (2005) highlight the potential for variability in female preference functions, not only between closely-related species (*H. chrysoscelis* versus *H. versicolor*), but also between distinct lineages within a single species (eastern versus western mtDNA lineages of *H. chrysoscelis*) as well as within a single lineage (Indiana versus Missouri populations of *H. chrysoscelis*).

Three important questions emerge upon considering the apparent variability illustrated in Figure 4. First, how robust are comparisons made across studies of different populations and reported by different researchers? While experimental methods of the three studies are similar in many ways, there is at least one important methodological difference worth considering. In the study by Morris and Yoon (1989), alternative stimuli were presented at sound pressure levels (93 dB SPL at 1.25 m) near the highest call amplitude reported for this species (Gerhardt 1975). In contrast, the current study and that by Gerhardt (2005) used playback amplitudes near the lower end of the natural range of variation (Gerhardt 1975). Call amplitude is positively correlated with body size in some frogs (e.g., in American toads, *Anaxyrus americanus*, Gerhardt 1975), though we presently lack data on this relationship in *H. chrysoscelis*. In addition, female selectivity for some spectral properties (e.g., a unimodal versus bimodal spectrum) can be level dependent in treefrogs (Gerhardt 1987; Gerhardt et al. 2007). Therefore, comparisons of preference functions for spectral frequencies across studies using different stimulus amplitudes are subject to the caveat that differences might arise due to variability in absolute sound level. Future studies should examine how preference functions for spectral properties vary with stimulus amplitude in *H. chrysoscelis*, bearing in mind that both the amplitude of male calls and the level dependence of female preferences could vary among populations.

Second, what physiological mechanisms might explain at a proximate level the observed population differences in selectivity for spectral properties? Frogs encode airborne sounds using two sensory papillae in the inner ear (reviewed in Gerhardt and Schwartz 2001; Gerhardt and Huber 2002). The amphibian papilla (AP) is tonotopically organized and tuned to relatively lower frequencies (e.g., < 1.5 kHz), whereas the basilar papilla (BP) lacks tonotopy and is tuned to a narrow range of higher frequencies (e.g., > 1.5 kHz). Previous studies have indicated some female preferences for spectral call properties can be predicted based on the tuning characteristics of one or the other papillae (reviewed in Gerhardt and Schwartz 2001). Therefore, one possible proximate-level hypothesis for the apparent variation in behavioral preferences for spectral properties among gray treefrogs is that different populations have differently tuned papillae. If this hypothesis were true, then we would predict papillae in *H. chrysoscelis* populations in Minnesota (and perhaps to a lesser extent, Missouri) to be tuned to higher frequencies than conspecifics in Indiana. Under this scenario, discrimination against calls with lower-than-average frequencies in Minnesota might be explained as lower-frequency calls being relatively less audible, thereby rendering them less attractive. Future studies could test this hypothesis directly using non-invasive procedures to measure auditory evoked potentials, such as the auditory brainstem response (Katbamna et al. 2006; Brittan-Powell et al. 2009).

Finally, can we explain the variability depicted in Figure 4 at an ultimate level in adaptive evolutionary terms? One possible adaptive explanation for the current patterns of geographic variation involves reproductive character displacement (Howard 1993). Two studies of closely related species pairs of North American treefrogs, including Cope's gray treefrogs (*H. chrysoscelis*) and eastern gray treefrogs (*H. versicolor*) (Gerhardt 1994) and green treefrogs (*H. cinerea*) and barking treefrogs (*H. gratioiosa*) (Höbel and Gerhardt 2003) have shown that females can be more selective for species recognition cues in areas of sympatry compared with allopatry (see also Márquez and Bosch 1997). Both *H. chrysoscelis* and *H. versicolor* have pulsed calls with similar bimodal spectra, but the average dominant frequencies of *H. versicolor* calls are typically about 200–300 Hz lower than those of *H. chrysoscelis* (Gerhardt 2001, 2005; Bee MA unpublished data). Females tested in our study and by Gerhardt (2005) were collected from syntopic populations of the two species or populations in close (e.g., 10 km) allopatry with *H. versicolor*. In contrast, *H. versicolor* reportedly does not occur as far south in Indiana as the populations where Morris and Yoon (1989) studied *H. chrysoscelis*, though the two species do occur in relatively close proximity

(e.g., 75 km) and the precise distributions of the two species in the state are still somewhat poorly known (Minton 1972, 2001). One hypothesis for the pattern of results depicted in Figure 4 is that in *H. chrysoscelis*, female selectivity favoring about average or higher-than-average calls in Minnesota and Missouri has evolved in response to selection against costly hybrid matings (Gerhardt et al. 1994) with *H. versicolor*. To the extent that Morris and Yoon's (1989) populations in Indiana have been historically isolated from contact with *H. versicolor*, females in their study may pay little or no costs associated with choosing heterospecific males with low-frequency calls and instead receive whatever benefits might accrue from mating with larger conspecific males.

While plausible, the adaptive explanation offered above has two weaknesses. First, there is broad overlap in the distributions of spectral frequency in both *H. chrysoscelis* and *H. versicolor* (see Fig. 2 in Gerhardt 2005). Frequency alone cannot be used to statistically (or acoustically) discriminate between the two species with any reliability. Moreover, there are other cues (e.g., pulse rate) that do not overlap between the two species and for which females exhibit much stronger preferences (Gerhardt 2001). Second, in all populations of *H. chrysoscelis* studied to date, preferences for call frequency are relatively weak. Furthermore, the preference for calls with higher frequencies in Gerhardt's (2005) *H. chrysoscelis* population was not maintained in the range of frequencies that overlap with *H. versicolor*. We would generally expect preference for good species recognition cues to be more robust than the frequency preferences so far observed in *H. chrysoscelis*. Nevertheless, it remains possible that spectral cues might function in combination with other species recognition cues (e.g., Poole and Murphy 2007), for example, by acting as an amplifier of species differences in other acoustic properties, such as pulse rate.

Conclusions

Multiple studies of *H. chrysoscelis* have now revealed broad geographic similarity across two recognized lineages in the spectral properties of advertisement calls. However, three studies of female preferences, when considered together, suggest possible geographic variation in population-level preference functions for spectral properties. Thus far, several different basic shapes of preference functions have been demonstrated in *H. chrysoscelis*, and at least some of these differ from that described for a closely-related sister-species, *H. versicolor*. However, given the uniform weakness of frequency preferences indicated in all three studies, we would conservatively conclude that the female preferences demonstrated in this and previous studies probably do not represent strong sources of selection on male calls or for males of a particular size class. Additional studies of the patterns of geographic variation in frequency preference functions using standardized methods, robust descriptions of underlying trait variation, and more purposeful sampling of both sympatric and allopatric populations (e.g., Gerhardt 1994; Márquez and Bosch 1997; Höbel and Gerhardt 2003) could shed some much needed light on the potential for evolutionary lability of female preference functions within this species and potential mechanistic and adaptive explanations for the patterns of preferences observed.

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Fig. 1.

Natural and synthetic calls. Shown here are oscillograms (top traces) and spectrograms (bottom traces) depicting the temporal and spectral structure of **a** a 680 ms natural call and **b** three 600 ms synthetic stimulus calls having frequencies that are 3 semitones (ST) below average (top), average (middle), and 3 ST above average (bottom). Oscillograms are depicted using the same time scales and each depicts a total duration of 680 ms.

Fig. 2.

Distributions of **a** fundamental frequency and **b** dominant frequency ($N = 50$ males) and scatterplots depicting the relationships between fundamental and dominant frequencies and **c**, **d** mass (fundamental frequency: Pearson $r = -0.40$, $p < 0.01$; dominant frequency: Pearson $r = -0.53$, $p < 0.01$; $N = 44$), **e**, **f** body length (snout-vent-length = SVL) (fundamental frequency: Pearson $r = -0.29$, $p = 0.06$; dominant frequency: Pearson $r = -0.39$, $p < 0.01$; $N = 44$), and **g**, **h** body condition (length-independent mass) (fundamental frequency: Pearson $r = -0.27$, $p = 0.08$; dominant frequency: Pearson $r = -0.35$, $p = 0.02$; $N = 44$).

Fig. 3.

Proportions ($\pm$ 95% binomial confidence intervals) of females selecting the alternative to the average call when both calls had equal amplitudes (filled circles) and when the average call was attenuated by 3 dB (open circles), 6 dB (open squares), or 9 dB (open triangles). Along the x-axis, tests of different alternatives to the average call are labeled with the absolute frequencies of the two spectral peaks in the alternative call as well as the frequency difference (in semitones, ST) between the average and alternative calls. Asterisks indicate significant difference from the null expectation of 0.5 in two-tailed binomial tests ($N = 20$ in all comparisons).

Fig. 4.

Comparisons of preference functions for *Hyla chrysoscelis* from **a** this study, **b** Morris and Yoon (1989), and **c** Gerhardt (2005) and for **d** *Hyla versicolor* from Gerhardt (2005). Two points connected by a line depict the relative proportions of females that chose each alternative in a two-choice test with the frequency values indicated along the x axis. Solid lines indicate significant differences ($p < 0.05$) in binomial tests as reported in this study or by Morris and Yoon (1989) and Gerhardt (2005); dashed lines indicate no statistical difference. In each pair of plots, data are shown for raw frequency values (in kHz, left) and standardized frequency values (in standard deviation units, right). For standardizing frequency values in **a–d**, we used the standard deviations **a** reported in this study, or standard deviations of **b** 200 Hz ($= 2 \times 100$ Hz reported for the low-frequency peak by Morris and Yoon (1989)); **c** 160 Hz (Gerhardt HC unpublished data) and **d** 200 Hz (Gerhardt HC unpublished data). For consistency across studies, supernormal stimuli have not been included and only data from choice tests in which the frequencies of both alternatives fell within the reported range of natural variation are included.

Table 1

Statistical results from two-tailed binomial tests of the outcomes of two-choice phonotaxis tests (Fig. 3) in which a call with an average frequency was paired with an alternative differing in frequency by ± 1 , ± 2 , or ± 3 semitones (ST).

Frequency difference	Relative amplitude of the average call	Binomial p	Effect size (g)
-3 ST	0 dB	< 0.01	0.45
-2 ST	0 dB	< 0.01	0.35
-1 ST	0 dB	0.82	0.05
+1 ST	0 dB	0.50	0.10
+2 ST	0 dB	0.26	0.15
+ 3 ST	0 dB	1.00	0.00
-2 ST	-3 dB	0.50	0.10
-2 ST	-6 dB	0.26	0.15
-2 ST	-9 dB	0.11	0.20
-3 ST	-3 dB	0.26	0.15
-3 ST	-6 dB	1.00	0.00
- 3 ST	-9 dB	0.01	0.30