
EFFECTS OF TEMPERATURE ON SPECTRAL PREFERENCES OF FEMALE GRAY TREEFROGS (*HYLA VERSICOLOR*)

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Abstract.—Males of many animal species produce conspicuous signals to attract mates; male treefrogs produce loud and persistent acoustic signals called advertisement calls. Frogs face an interesting challenge in that temperature can differentially impact signal production and perception, leading to a mismatch between sender and receiver. For instance, female Green Treefrogs (*Hyla cinerea*) exhibit temperature-sensitive preferences for the frequency of the advertisement call, potentially resulting in interspecific hybridization at low temperatures. Considering climate predictions by the Intergovernmental Panel on Climate Change, we investigated whether, and by how much, temperature modifies female preferences for natural variation in spectral properties of male advertisement calls. The mate-choice preferences of the Gray Treefrog (*H. versicolor*) have been extensively studied; females prefer calls with standard bimodal frequency peaks of 1100 and 2200 Hz over calls with higher and lower frequencies. These preferences were determined at 20° C, but the dominant frequency of calls is positively correlated with temperature. Using two-speaker choice experiments, we tested the hypothesis that acoustic preferences of female *H. versicolor* for the frequency of male advertisement calls vary based on ambient temperatures (15, 20 and 25° C). We found that female preferences based upon frequency are, at best, only moderately temperature dependent. We discuss the possible neurophysiological basis for the seeming lack of temperature coupling in this aspect of the Gray Treefrog communication system, and conclude that mate-choice decisions based on frequency will not be significantly impacted by modest (2° C) changes in environmental temperatures.

Key words.—climate change; communication; frequency; phonotaxis

INTRODUCTION

Climate change is altering ecological systems across multiple levels of biological organization (Walther 2010). The mean global air temperature is projected to increase by 2–4° C in the next 50–100 y (Intergovernmental Panel on Climate Change 2007) causing the climate in some portions of the mid-western United States to more closely resemble Texas (Karl et al. 2009). More recent modeling predicts that by the end of the 21st Century, almost all areas of North America will experience increases of at least 5° C in the warmest daily maximum temperature (IPCC 2014). For anuran amphibians, forecasts predict significant population declines and extinctions resulting from temperature-dependent changes in growth and reproduction (McCallum 2010) or direct mortality resulting from increased metabolic rates and reduced water availability (Bickford et al. 2010). Although organisms may be able to respond to changing climate conditions by physically tracking preferred microclimates utilizing morphological and behavioral adaptations related to dispersal or migration, or tolerating changing conditions using physiological and behavioral adaptations related to thermoregulation and thermotolerance (Sears and Angilletta 2011), there is still the potential for climate to

have profound consequences through its effects on other temperature-dependent processes. Because amphibians are widely considered to be an indicator species of environmental change (Semlitsch 2003), understanding how the breeding system of frogs could be affected by temperature changes will help better understand the potential sub-lethal impacts of climate change.

Many muscular and neurological functions are temperature-dependent in ectothermic organisms (Yamaguchi et al. 2008). These physiological effects can translate into temperature-dependent aspects of acoustic communication systems. First, temperature can affect production of signals: advertisement calls with a pulsatile structure are produced at an increased repetition rate, with higher dominant frequencies and shorter pulse durations as temperature increases (Gerhardt 1994; Lüddecke and Sánchez 2002). The dominant frequency of anuran calls is most strongly affected by body size, and many anuran species show no temperature-dependence in call frequency (Gerhardt and Huber 2002). The positive relationship between temperature and frequency observed in some frog species may result from higher air pressures generated in the vocal system of warmer frogs (Martin 1971). Second, temperature can impact the perception of acoustic signals by receivers. In anuran amphibians, one of the two hearing

organs in the peripheral auditory system functions in a temperature-dependent fashion. The basilar papilla detects high frequency sounds and functions in a temperature-independent fashion while the amphibian papilla detects lower frequency sounds and operates in a temperature-dependent fashion (Mudry and Capranica 1987; Stiebler and Narins 1990).

Although there are documented examples of temperature coupling, in which temperature-dependent changes in both signalers and receivers can result in coordinated mating behaviors in environments with seasonal variation in temperature, we do not have a broad body of descriptive work examining the influence of ecologically relevant temperature variation on female acoustic preferences. In Eastern Gray Treefrogs (*Hyla versicolor*), the pulse repetition rate and the preference for it are tightly coupled across a range of temperatures (Brenowitz et al. 1985; Gerhardt and Doherty 1988; Gerhardt 2005a). Rather than coordinating mating behavior in an adaptive fashion, however, temperature coupling may simply result from common effects of temperature on song and preferences (Ritchie et al. 2001; Greenfield and Medlock 2007), and we know of instances in which the male signal and female preference are uncoupled (Lewis and Narins 1999). For example, Gerhardt and Mudry (1980) demonstrated in the Green Treefrog (*H. cinerea*), that the female preference for dominant frequency is positively linearly related to temperature, while the dominant frequency of the male call is not. Narins (2001) pointed out that, regardless of the degree to which call properties are temperature-dependent (not at all or weakly correlated), the mismatch in the communication system arises because thermal sensitivities vary between sender (spectral components of call) and receiver (tuning of low-frequency receptors in the inner ear).

Mismatches between senders and receivers can have profound negative impacts on successful reproduction. In Green Treefrogs, the magnitude of the mismatch is quite striking. Gerhardt and Mudry (1980) found that an ambient temperature drop of 7° C corresponded with a 400 Hz downward shift in the average female preference for dominant frequency of the male advertisement call, a change that increased the likelihood of hybridization with a sister species, the Barking Treefrog (*H. gratiola*). Communication systems have probably evolved to account for slight differences in the body temperatures of signalers and receivers; for instance, *H. versicolor* within a breeding aggregation experience microclimates that vary by up to 4° C on a given night (Ptacek 1991). However, this local environmental heterogeneity might exacerbate climate-induced mismatches in the communication system. Mismatches, such as the one described in *H. cinerea*, could have significant consequences for the population, the community, and the

ecosystem if climate change leads to prolonged mismatches between senders and receivers.

In this study, we examined whether the decoupling of signal and preference is a general phenomenon as it relates to spectral composition of an acoustic mate-attraction signal. We explored this hypothesis by testing the frequency preferences of *H. versicolor* at different temperatures. As in Green Treefrogs, the dominant frequency of the *H. versicolor* call is positively correlated with temperature (Gerhardt 2005b), although the magnitude of the effect is only 12 Hz per 1° C change in temperature (Gayou 1980). Based on the temperature-dependent preferences exhibited by females of *H. cinerea* and the relationship between temperature and frequency in advertisement calls of *H. versicolor*, we predicted that, relative to a call with characteristics typical of one recorded from our population at 20° C, female *H. versicolor* would prefer lower-frequency calls at 15° C and higher-frequency calls at 25° C.

MATERIALS AND METHODS

Playback protocol.—We collected mated pairs of *H. versicolor* by hand between 2000 and 2300 during May–June of 2008 and 2009 from Baskett Wildlife Research Area, Boone County, Missouri, USA. We then transported frogs to the laboratory at the University of Missouri in ventilated plastic boxes and stored them on ice to prevent females from ovipositing. The next morning, female frogs were allowed to acclimate to the desired testing temperature in an incubator for a minimum of 30 min. We set one incubator at 20° C and adjusted a second incubator to 15 or 25° C ($\pm 0.5^\circ$ C). Prior to the first test at a new temperature, we checked the deep body temperature of each frog using a cloacal thermometer. We allowed females more than 2° C from the desired temperature to acclimate within the incubator until their body temperature fell within the allowable temperature range.

All playback experiments were conducted in a temperature-controlled, semi-anechoic chamber described in Gerhardt (1994). We briefly confined each female in a 10-cm diameter, acoustically transparent release cage positioned midway between two speakers until each alternative stimulus had been broadcast three times in an alternating fashion, at which time we remotely removed the top of the cage. We observed the behavior of the female from outside the chamber using a closed-system, IR-sensitive camera. We continued playbacks until a female moved to within 10 cm of a speaker after having shown phonotactic orientation behavior such as head scanning and zig-zag walking (Rheinlander et al. 1979), at which time we scored her as having made a Choice. We recorded a No Response if females did not leave the release cage within 2 min or make a choice within 5 min. There was a time-out of at

TABLE 1. Acoustic properties of synthetic stimuli used to test the frequency preferences of female *Hyla versicolor* at three different temperatures. Pulse rate and pulse duration are temperature-corrected to the population mean at the collection site based on observations (Gayou 1980). Pulse rise- and fall-times were 80% and 20% of the total pulse duration, respectively. The inter-pulse interval was the same as the pulse duration, resulting in a 50% pulse duty cycle in all stimuli. Call duration and call period were not temperature corrected, but kept constant across temperatures.

Call Properties	15° C	20° C	25° C
Pulse rate (pulses per s)	15	20	26
Pulse duration (ms)	33.3	25	19.2
Pulse rise-time (ms)	26.64	20	15.36
Pulse fall-time (ms)	6.66	5	3.84
Inter-pulse interval (ms)	33.3	25	19.2
Call duration (ms)	965 (15)	975 (20)	980 (26)
(# pulses)			
Call period (s)	4.0	4.0	4.0

least 5 min between the response of an individual female in one test and the beginning of another test at the same temperature. There is no evidence that the exposure of a female to, or choice of, a particular stimulus impacts her subsequent choices under these conditions (Gerhardt et al. 2000). Even though control tests indicate that no side biases exist in the chamber (Gerhardt et al. 2000), we switched stimuli periodically between speakers. When the frogs completed all tests or stopped responding, we placed them in a ventilated container and returned them to their pond of origin within two days.

Acoustic stimuli.—We synthesized 16-bit acoustic stimuli using custom-designed software. The advertisement calls of *H. versicolor* are comprised of a series of short pulses. The fine-temporal properties of the advertisement call are strongly temperature-dependent and females are highly selective for appropriate pulse-repetition rates (Gerhardt 2005a). We synthesized calls with average pulse-repetition rates for each temperature based on the regression coefficient determined by Gayou (1980, 1984; Table 1). Although they are also temperature dependent, call duration and call-repetition rates show much higher levels of within- and between-male variation. To control for strong female preferences for increased calling effort (Gerhardt et al. 2000), we kept constant (to within 15 ms) the total call duration and call-repetition rate of stimuli used at different temperatures. The spectral energy of advertisement calls has a bimodal distribution, and the two peaks are harmonically related (the high-frequency peak is twice the frequency of the low-frequency peak; Fig. 1a-c). Because the average values of these frequency components in our study population were 1100 and 2200 Hz, we chose to use these values in all Standard stimuli. Even though the low-frequency peak is normally 6-10 dB less than the high-frequency peak

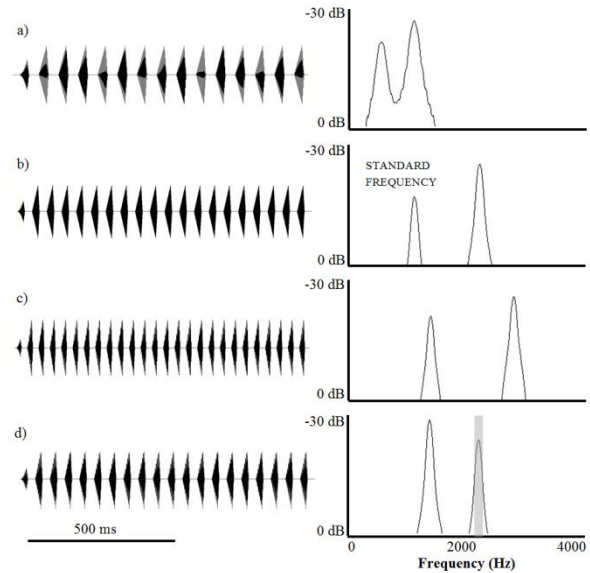


FIGURE 1. Oscillograms and power spectra of stimuli synthesized for the first (panels a-c) and second (panel d) experiments. Three representative stimuli are shown, exhibiting temperature-dependent variation in pulse duration and pulse repetition rate (left images), as well as variation in frequency (right images). a) 15° C and 550/1100 Hz; b) 20° C and 1100/2200 Hz, the Standard call; c) 25° C and 1400/2800 Hz; d) The relative amplitude of the two spectral peaks was reversed in 2009, such that the low-frequency peak was louder than the high-frequency peak. The high-frequency peak was always kept at 2200 Hz, as emphasized by the gray shading. The power spectrum shows the 1375/2200 Hz stimulus at 20° C.

(Fig 1a-c), these are the frequencies that should stimulate the temperature-dependent amphibian papilla, and they have a large effect on signal attractiveness even when attenuated 24–30 dB relative to the high-frequency peak (Gerhardt 2005b). Thus, as in natural calls, all synthetic calls contained two spectral components.

In our local population, the values of the low-frequency spectral component ranged between 1000 and 1400 Hz (Gerhardt 2005b), so for our first experiment, we designed test stimuli possessing low-frequency peaks at 550, 950, 1000, 1050, 1100, 1200, 1300, 1400, and 1500 Hz. In all test stimuli, the high-frequency peak was 6 dB louder than the low-frequency peak, which simulates the natural distribution of spectral energy in an advertisement call. Also, as in natural calls, we maintained the 2:1 harmonic relationship between the two peaks and changed the high-frequency peak in correspondence with the low-frequency peak (Fig. 1a-c). These nine different frequencies were tested at each of three temperatures, for a total of 27 different tests.

Because the frequencies of the two spectral components roughly match the sensitivities of the two inner ear organs (Gerhardt and Schwartz 2001; Gerhardt 2008) and the higher peak was twice as loud as the lower peak, we considered it likely that the basilar papilla (sensitive to high frequency, not temperature dependent)

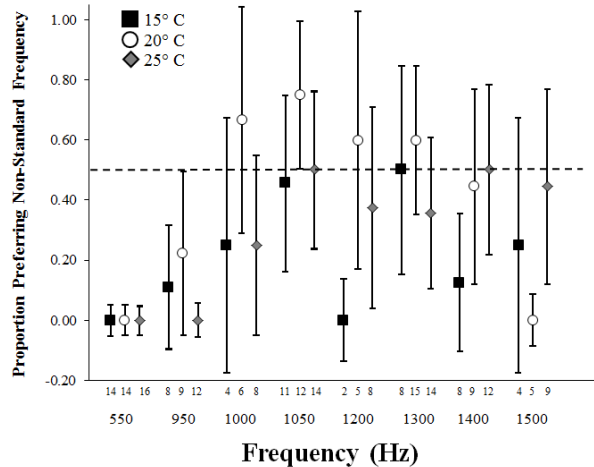


FIGURE 2. Temperature-dependent frequency preferences exhibited by *Hyla versicolor* when the high-frequency peak of stimuli is emphasized. The graph shows the proportions of females choosing stimuli with varying low-frequency peaks over the standard-frequency call (1100 and 2200 Hz) at three different temperatures. The relative amplitude of the two peaks is similar to those found in natural calls, with the high-frequency peak being 6 dB louder than the low-frequency peak. The X-axis indicates the value of the low-frequency peak in each stimulus. Error bars represent 95% confidence intervals.

was much more highly stimulated than the amphibian papilla (sensitive to low frequency, temperature dependent) by the original set of test stimuli. Thus, in our second experiment, we synthesized stimuli in which the low-frequency peaks were 6 dB louder than the high-frequency peaks. Also, to control for stimulation of the basilar papilla, we kept the high frequency peak constant at 2200 Hz while varying the low-frequency peak (Fig. 1d). These stimuli should have increased our ability to observe temperature-dependent function in the amphibian papilla, while at the same time maintaining two-tone facilitation. We also used somewhat different values for low-frequency peaks (550, 825, 1100, 1375, 1650 Hz), as they were harmonically related (275 Hz fundamental) to the normal 1100 and 2200 Hz frequency peaks. In the few instances it has been examined, there are very little data to indicate that harmonic structure is important for female mate-choice (Bodnar 1996; Akre et al. 2014). Our choice of low-frequency values was based on the observation that spectral components are usually harmonically related in frog species that produce calls with multiple frequency peaks.

Two-speaker choice tests.—We combined standard and alternative call stimuli in a 16-bit digital stereo file with a sampling rate of 20 kHz. Acoustic stimuli were output from a desktop computer (Dell, Austin, Texas, USA) with a SoundMAX ADI 198x Integrated Audio Device sound card (Analog Devices, Inc., Norwood, Massachusetts, USA), amplified using an Alesis RA150 amplifier (Alesis, Cumberland, Rhode Island, USA), and

broadcast using two Analog-Digital-Systems 200 loudspeakers (ADS, Wilmington, Massachusetts, USA). The speakers were separated by 2 m and facing one another. We equalized the signal strength of alternative stimuli at the female release point midway between the speakers at 85 dB SPL (re 20 μ Pa, C-weighting, fast transform) with a Larson Davis Laboratories Model 800B SPL meter (Larson Davis, Depew, New York, USA). Calls had a period of 4 s, and alternated so that neither stimulus led or lagged the other.

We tested female preferences for the frequency of the call by comparing their responses to a standard call (1100 + 2200 Hz) versus the alternatives described above. We ran each test at three temperatures (15, 20, and 25° C). Before beginning a series of tests, we adjusted the temperature of the chamber to within $\pm 1^\circ$ C of the set point. Over the course of the day, series of tests were generally run in the order of 15, 20, 25° C because it was easier to warm the chamber than it was to cool it. Although temperature treatment and time of day are perfectly collinear, and are potentially confounded, there is no evidence that females exhibit diurnal patterns in their responsiveness to acoustic stimuli (Zeyl and Laberge 2011). We present results of the tests as the proportion of females that chose the alternative over the standard (1100 + 2200 Hz) stimuli and 95% confidence intervals. We considered a preference to be statistically significant if at least 14/20 chose a particular stimulus (P -value = 0.039, two-tailed binomial test). This criterion translates to 95%-credible limits of $\geq 50\%$ if most females chose the alternative and $\leq 50\%$ if most females chose the standard call. Differences among temperature were tested non-parametrically using a Cochran Q test with $\alpha = 0.05$.

RESULTS

Experiment #1.—Forty-one females of *H. versicolor* responded in 257 tests. Females responded in an average of 5.6 choice tests. There were 67 responses at 15° C (overall response rate of 71%), 85 responses at 20° C (overall response rate of 79%), and 105 responses at 25° C (overall response rate of 91%). Because of the relatively small sample sizes at the end of the season, and the decision to change the acoustic stimuli the following year, we did not conduct formal statistical testing with this set of data. However, visual appraisal of the data indicates a couple of patterns (Fig. 2). First, the confidence intervals are broadly overlapping at each frequency, indicating no effects of temperature on female preference. Females strongly avoided stimuli with extremely low, low-frequency peaks (550 and 950 Hz) at all temperatures. Second, although the preference functions generated at each temperature were roughly unimodal, with a most-preferred frequency somewhere near the population average of 1100 Hz, a rough

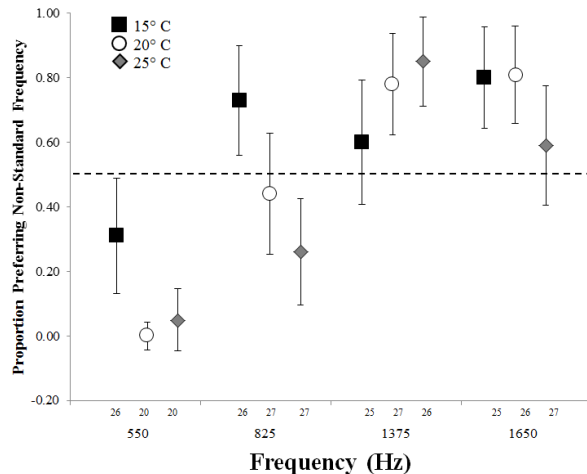


FIGURE 3. Temperature-dependent frequency preferences exhibited by *Hyla versicolor* when the low-frequency peak of stimuli is emphasized. The graph shows proportions of females choosing stimuli with varying low-frequency peaks over the standard-frequency call (1100 and 2200 Hz) at three different temperatures. The X-axis indicates the value of the low-frequency peak, which is 6 dB louder than the high-frequency peak. Sample sizes are indicated beneath each test result. Error bars represent 95% confidence intervals.

comparison of the preference functions generated at 20 and 25° C suggests that the preference function is broader at warmer temperatures.

Experiment #2.—Thirty-five females of *H. versicolor* responded in 302 tests. Five females made choices in all 12 tests and females responded in an average of 8.6 tests. There were 102 responses at 15° C (overall response rate of 86%), 100 responses at 20° C (overall response rate of 98%), and 100 responses at 25° C (overall response rate of 93%). The data reveal temperature-dependent preferences for lower-than-average frequencies, but not for higher frequencies (Fig. 3). Visual inspection reveals that, in response to the 550 and 825 Hz calls, there was an inverse relationship between temperature and the preference for the low-frequency alternative: females at low temperatures were more likely to choose these calls than females at higher temperatures. There was a significant effect of temperature for the 825/2200 Hz versus the 1100/2200 Hz standard call ($Q_2 = 9.500$, $n = 22$, $P = 0.009$). There was no significant difference across the temperature treatments for the standard call versus the 550/2200 Hz stimulus ($Q_2 = 2.000$, $n = 11$, $P = 0.368$). However, post-hoc pair-wise comparisons, without correction for multiple comparisons, indicated a significant difference between responses at 15° and 20° C ($Q_1 = 5.00$, $n = 16$, $P = 0.025$). There were no significant differences between the 20° and 25° C temperatures ($Q = 1.000$, $P = 0.317$) or the 15° and the 25° C temperatures ($Q = 0.333$, $P = 0.564$). Although there appears to be a trend whereby preferences for the

1375-Hz alternative were stronger at higher temperatures, there were no significant differences across the temperatures for the standard frequency versus the 1375 Hz stimulus ($Q = 1.143$, $P = 0.56$) or the 1650 Hz stimulus ($Q = 3.250$, $P = 0.20$).

DISCUSSION

The results of our study indicate that female preferences based upon frequency are at best only moderately temperature dependent. When tested at species-typical relative amplitudes in our first experiment, there is some indication that preferences for higher frequency low-frequency peaks are broader at warmer temperatures. In other words, females at warmer temperatures appeared to be more likely to choose high-frequency stimuli. When tested in a manner that emphasizes the low-frequency spectral component, in our second experiment, there is no evidence of this shift at high frequencies, but rather a greater permissiveness for low frequencies at low temperatures.

While this study does not provide strong evidence of temperature coupling in the spectral domain for this species, it would not be correct to say that the communication system is maladaptively decoupled as it is in *H. cinerea* (Gerhardt and Mudry 1980). Rather, it appears that the preference function for frequency is fairly broad. Although the distribution of mean values of low-frequency spectral peaks recorded from advertisement calls is fairly narrow (1100–1250 Hz; Gerhardt 2005b), females are almost equally permissive to signals with low-frequency peaks ranging between 1000 and 1400 Hz at 20° C. Although shifted upward somewhat, this 400 Hz range of acceptable frequencies is also observed at 25° C. In a frequency-preference study where the high-frequency spectral component was simultaneously modified (maintained at twice the low-frequency value), females did not discriminate against high-frequency calls until they were 1400/2800 Hz or above (Gerhardt 2005b). In short, female preferences for frequency are not strong in this species (Gerhardt 2005b; this study). Even if average call frequencies shift upward in response to increases in ambient temperatures ($8^\circ \text{C} \times 12 \text{ Hz}/^\circ \text{C} = 96 \text{ Hz}$), they will almost certainly remain within the range of acceptable frequencies for female mate choice. In addition, females of *H. versicolor* do not use frequency differences to discriminate between conspecifics and the closely related congener, Cope's Gray Treefrog (*H. chrysoscelis*), so the risk of hybridization is low (Gerhardt 2005b).

Treefrogs detect sounds in their environment using two different hearing organs, the basilar papilla and the amphibian papilla (Schoffelen et al. 2008). The basilar papilla functions independently of temperature, as it appears to be mechanically tuned via the tectorial

membrane. The amphibian papilla responds to acoustic stimulation in a temperature-dependent fashion, as the hair cells are electrically tuned. In an attempt to avoid complications posed by both spectral peaks stimulating both auditory organs to some extent (Gerhardt 2005b), we designed test stimuli that would never be observed in nature. We used a low-frequency spectral component varying independently of a constant, high-frequency spectral component, which itself was 6 dB quieter than the low-frequency peak. Even in this scenario, we observed very little evidence of temperature-dependent frequency preferences. One possible explanation is simply that the calls of *H. versicolor* do not strongly stimulate the temperature-dependent amphibian papilla. In recordings from the auditory nerves of American Bullfrogs (*Lithobates catesbeianus*) and Pacific Chorus Frogs (*H. regilla*), the amount of temperature-dependent frequency shift decreased as frequency increased and stimulation of fibers in the amphibian papilla decreased (Stiebler and Narins 1990; van Dijk et al. 1990). Interestingly, spike rates increased considerably as temperature was raised (van Dijk et al. 1990), and tone-response thresholds decreased with increasing temperatures (Stiebler and Narins 1990), perhaps explaining the positive correlation between temperature and response rates that we observed. Thus, we conclude that in natural choruses, temperature will have little to no impact on female mate choice based on frequency.

Indirect impacts of climate change on amphibian reproductive behavior should not be ruled out. The temporal parameters of the calls, such as pulse-repetition rate, calling rate, and call duration exhibit higher thermal sensitivities than spectral traits (Gayou 1984; Gerhardt and Huber 2002). Because higher latitudes are predicted to experience greater warming over the next century (IPCC 2014), and the species range of *H. versicolor* extends over a wide range of latitudes (Canada to south-central Texas), future studies should examine whether patterns of temperature-dependent preferences are similar in populations located in the northern and southern portions of the range of the species. It is possible that populations adapted to different climatic regimes will respond differently to temperature variation. For instance, in the Andean Frog (*H. labialis*), males from the coldest climate produced calls exhibiting the least thermal sensitivity in pulse-repetition rate, even though they called across the widest temperature range (Lüddecke and Sanchez 2002). In general, the range of thermal tolerance increases with latitude (Navas et al. 2008). Finally, changes in temperature during larval development could potentially impact signal production and preferences in adult animals (Grace and Shaw 2004). Although they did not describe female preferences, a ground-breaking study using data collected over a 23-y period indicates that males of the Puerto Rican Coqui Frog (*Eleutherodactylus coqui*) are currently producing

calls of higher frequency and shorter duration, with values closely matching the magnitude of the rise in temperature over that period (Narins and Meenderink 2014). Future studies should attempt to predict temperature-induced disruptions in the communication systems of anurans living at different latitudes and elevations.

An understanding of how *H. versicolor* mating behavior is affected by local environmental heterogeneity within a breeding season is imperative for understanding how wild populations could be affected by global climate change. It is possible that the environment may be altered to the extent that breeding is reduced below sustainable levels. Therefore, a better understanding of the chronic and sub-lethal effects of warmer temperatures on breeding populations of amphibians will help biologists and managers to effectively prioritize conservation efforts.

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