Female preferences for the spectral content of advertisement calls in Cope's gray treefrog (*Hyla chrysoscelis*)

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Abstract

Amphibians have inner ears with two sensory papillae tuned to different frequency ranges of airborne sounds. In frogs, male advertisement calls possess distinct spectral components that match the tuning of one or both sensory papillae. Female preferences for the spectral content of advertisement calls can depend on signal amplitude and can vary among closely related lineages. In this study of Cope's gray tree frog (*Hyla chrysoscelis*), we investigated the amplitude dependence of female preferences for the spectral content of male advertisement calls, which have a "bimodal" spectrum with separate low-frequency (1.25 kHz) and high-frequency (2.5 kHz) components. In two-alternative choice tests, females generally preferred synthetic calls with bimodal spectra over "unimodal" calls having only one of the two spectral components. They also preferred unimodal calls with a high-frequency component over one with the low-frequency component. With few exceptions, preferences were largely independent of amplitude across both a 30 dB range of overall signal amplitude and an 11 dB range in the relative amplitudes of the two spectral components. We discuss these results in the context of evolutionary lability in female preferences for the spectral content of advertisement calls in North American treefrogs in the genus *Hyla*.

Keywords Acoustic communication, Sensory perception, Signal recognition, Spectral processing, Vocal communication

Introduction

Anuran amphibians (frogs and toads) are important neuroethological study systems for elucidating how natural sounds, particularly vocalizations, are processed by the auditory system to guide behavioral decisions (Feng and Ratnam 2000; Feng and Schul 2007). In most anuran species, males produce simple vocalizations that have stereotyped and species-specific spectral and temporal properties and that elicit robust behavioral responses in both male and female conspecifics (Gerhardt 1994a; Gerhardt and Huber 2002; Gerhardt and Bee 2007; Wells and Schwartz 2007; Bee et al. 2016). The advertisement calls that males produce to attract gravid females and repel rival males play key roles in species recognition and sexual selection. Identifying how behaviorally relevant acoustic features of advertisement calls and other vocal signals are processed by the anuran auditory nervous system has been a key focus of neuroethological research for decades (Fritzsch et al. 1988; Feng et al. 1990; Gerhardt and Huber 2002; Narins et al. 2007; Rose 2014; Ponnath et al. 2022). One of the pioneers and leaders in research on the neural basis of sound pattern recognition in anurans, and in particular the neural coding of an advertisement call's spectral content, was Dr. Albert Feng, to whom this article is dedicated.

The anuran peripheral auditory system is specialized for encoding the frequency spectrum of species-specific vocalizations (Capranica 1965, 1966; Frishkopf et al. 1968; Feng et al. 1975). Anurans have two sensory organs in their inner ear — the amphibian papilla and the basilar papilla — that encode different ranges of airborne sound frequencies (Lewis and Lombard 1988; Zakon and Wilczynski 1988; Lewis and Narins 1999; Simmons et al. 2007). The frequency tuning of one or both papillae typically matches frequencies emphasized in the advertisement calls produced by conspecific males (i.e., a peripheral matched filter) (Gerhardt and Schwartz 2001; Simmons 2013). The amphibian papilla is tonotopically organized and tuned to low and intermediate frequencies, whereas the basilar papilla is broadly tuned to higher and similar frequencies (Moffat and Capranica 1974; Capranica and Moffat 1975; Feng et al. 1975; Zakon and Wilczynski 1988). Early studies of North American bullfrogs (*Rana catesbeiana*) by Capranica (1965, 1966) revealed that spectral content is an important cue for call recognition in this species. Males produce advertisement calls with a "bimodal" frequency spectrum having simultaneous low-frequency and high-frequency acoustic energy occurring in the ranges of the amphibian and basilar papillae, respectively. The evoked vocal responses of males were more robust when both spectral components were present in calls and call-like stimuli. More recent studies of several species of treefrog in the genus *Hyla* (Hylidae) have shown broadly similar results. In species with calls having bimodal spectra, females typically prefer bimodal calls with both spectral components over bimodal calls having one spectral peak attenuated or “unimodal” calls having only one spectral peak (Gerhardt 1974, 1981a, b, 2005; Gerhardt et al. 2007; Bee 2010; Lee et al. 2017).

As in other vertebrates, auditory nerve afferents from both the amphibian and basilar papillae exhibit simple, V-shaped frequency tuning curves (e.g., Feng et al. 1975), whereas auditory neurons in the central auditory system can exhibit much greater complexity in their spectral tuning (e.g., Feng et al. 1990). Extensive work by Albert Feng and his colleagues aimed to discover how the frequency tuning evident at the periphery is modified centrally along ascending stages of the auditory system (Fuzessery and Feng 1981, 1982, 1983a, b; Gooler et al. 1993, 1996; Xu et al. 1994; Zhang and Feng 1998; Zhang et al. 1999; Goense and Feng 2005). This body of work, and in particular a key series of studies by Fuzessery and Feng (1981, 1982, 1983a, b), helped elucidate how neural selectivity for the spectral content of conspecific calls increases at each successive level of the ascending auditory system (reviewed in Feng et al. 1990; Hall 1994). For example, in the dorsal medullary nucleus (DMN) and superior olivary nucleus (SON) most neurons exhibit unimodal, V-shaped excitatory tuning curves similar to those of auditory nerve fibers, whereas some SON neurons, but not DMN neurons, exhibit bimodal, W-shaped tuning curves with enhanced sensitivity in two distinct frequency regions (Fuzessery and Feng 1983a). The inferior colliculus (IC) exhibits a much wider range of tuning curve shapes, including a higher proportion of bimodal, W-shaped tuning curves with two separate regions of increased sensitivity at frequencies near those emphasized in conspecific advertisement calls, with such neurons becoming more frequent and having more complex tuning in the auditory thalamus (Fuzessery and Feng 1982, 1983b). Combination-sensitive auditory neurons, found first in the inferior colliculus and becoming more prominent in the thalamus, often fire only or maximally when presented with separate low-frequency and high-frequency spectral components that match the spectral components emphasized in the species’ advertisement calls (Fuzessery and Feng 1982, 1983b; see also Megela 1983; Mudry and Capranica 1987a, b; Lee et al. 2017).

According to Hall (1994), combination-sensitive neurons exhibit characteristics of the neuronal “mating call detector” originally envisioned by Frishkopf et al. (1968), though such an interpretation has been questioned (Simmons 2013) and is too simple to be entirely consistent with results from some behavioral studies. For example, in some hylid treefrog species a bimodal frequency spectrum is not required for call recognition, as females respond readily to synthetic unimodal calls lacking the species-typical bimodal frequency spectrum, even at low signal amplitudes, for example less than 50 dB (Gerhardt 1974, 2008; Nityananda and Bee 2011, 2012). Moreover, preferences for spectral content can be nonlinear and vary as a function of both overall signal amplitude as well as the relative amplitudes of different spectral components (Gerhardt 1974, 1981a, b, 2005; Gerhardt et al. 2007). Some level-dependent nonlinearity in preferences probably results from overlap in frequency tuning between the amphibian and basilar papillae that occurs at high sound amplitudes (Gerhardt and Schul 1999; Gerhardt 2005; Schrode et al. 2014). Lastly, between closely related species in the same genus, and even between genetically distinct lineages within a single species, females can exhibit divergent preferences for the spectral content of advertisement calls (Gerhardt 1974, 1981a, b, 2005; Gerhardt et al. 2007; Bee 2010). The evolutionary basis for this diversity in signal recognition and preferences, and its underlying divergent mechanisms, remain unknown. Thus, while considerable progress has been made in understanding neural adaptations that give rise to a general behavioral selectivity for the spectral features of conspecific advertisement calls in anurans, this understanding remains inadequate to fully explain intra- and interspecific differences in the preferences females exhibit for spectral content.

As Gerhardt (2008, p. 2610) noted in a study of spectrally dependent preferences in treefrogs, “quantitative behavioral experiments, which explore the multi-variate acoustic space of signals that best elicit responses from the whole animal, are an important complementary step to understanding how auditory systems recognize biologically significant signals.” In this vein, we investigated female preferences in Cope’s gray treefrog (*H. chrysoscelis*) for the spectral content of male advertisement calls and the extent to which spectral preferences vary as a function of sound amplitude. Male Cope’s gray treefrogs produce advertisement calls that consist of a train of about 30 pulses having a species-specific pulse rate near 50 pulses/s (Ward et al. 2013). Each pulse in a natural call has a bimodal frequency spectrum consisting of two harmonically related spectral components centered close to 1.25 kHz and 2.5 kHz, with the low-frequency spectral component having an average amplitude of -11 dB (range: -1.7 to -24.2 dB) relative to the high-frequency spectral component (Ward et al. 2013). Audiograms based on multiunit recordings from the inferior colliculus (Hillery 1984) and the auditory brainstem response (Schrode et al. 2014) have the expected W-shape indicative of the joint tuning of the amphibian and basilar papillae, which are tuned to the low-frequency and high-frequency spectral components of the advertisement call, respectively. Females prefer calls having both frequency components, but readily respond to synthetic calls having one or the other component (Gerhardt and Doherty 1988; Gerhardt 2005; Gerhardt et al. 2007; Bee 2010; Nityananda and Bee 2011, 2012). In the Western clade of *H. chrysoscelis* (Booker et al. 2022), females also prefer high-frequency unimodal calls over low-frequency unimodal calls (Bee 2010). However, the extent to which female preferences for bimodal over unimodal calls, and for calls with the high versus the low spectral component, depend on sound amplitude has not been investigated in depth. Therefore, we used female phonotaxis in two-alternative choice tests to investigate amplitude-dependent preferences for calls with the species typical bimodal spectrum versus calls with a unimodal spectrum (Experiment 1) and for unimodal calls with a high-frequency versus a low-frequency spectral component (Experiment 2).

Methods

Subjects

We used 75 gravid females of the Western clade of *H. chrysoscelis* (Booker et al. 2022) as subjects for this study. We collected subjects in amplexus between 2100 h and 0100 h during the 2018 and 2019 breeding seasons (May – June) from wetlands at the Carver Park Reserve (Carver County, MN, USA) and the Hyland Lake Park Reserve (Hennepin County, MN, USA). Populations of *H. chrysoscelis* within these wetlands are in allopatry with a visually indistinguishable sister species, the eastern gray treefrog *Hyla versicolor*. Each pair was collected in a separate small plastic container and transported back to the laboratory on the St. Paul campus of the University of Minnesota – Twin Cities. All animals were provided with aged tap water and they were maintained at approximately 4ºC until testing to delay egg-laying and extend the window during which females are behaviorally responsive to acoustic stimuli (Gerhardt 1995). Prior to testing, pairs were transferred to a temperature-controlled incubator so that their body temperature could reach 20 ± 1°C over the course of a 30 to 40 min acclimatization period. All experiments were conducted at 20 ± 1°C because female responses to calls are temperature-dependent (Gerhardt 1978), and 20°C is close to the average nighttime temperature recorded during active choruses in local populations. Subjects were released at their site of capture after testing was completed (usually within 24-48 hours of capture).

Acoustic stimuli

We generated synthetic acoustic stimuli (44.1 kHz, 16 bit) using custom scripts written in MATLAB version 2016b (MathWorks, Natick, MA, USA). Acoustic stimuli were modeled after the natural advertisement calls and calling behavior of *H. chrysoscelis* recorded from our study population, after adjusting values to a common temperature of 20°C using linear regression (Ward et al. 2013). All stimuli generated for this study consisted of a sequence of synthetic calls presented at a constant call rate of 11 calls/min to simulate a naturally calling *H. chrysoscelis* male. Each call consisted of 30, 10-ms pulses repeated at a species-specific pulse rate of 50 pulses/s and with a pulse duty cycle of 50%. The amplitude envelope of each pulse was shaped with species-typical rise and fall times (3.1-ms inverse exponential rise time; 5.4-ms exponential fall time). Across stimuli, we manipulated whether the pulses were bimodal in having two phase-locked spectral components (1.25 kHz *and* 2.5 kHz) or unimodal in having just one of the two spectral components as the sole carrier frequency (1.25 kHz *or* 2.5 kHz). We created three versions of the bimodal stimulus that differed in the amplitude of the low-frequency component relative to that of the high-frequency component (0 dB, -5 dB, and -11 dB; Table 1). During any given two-alternative choice test, the two stimulus sequences were broadcast in an alternating fashion such that there were equal intervals of silence before and after each call. We also created two separate versions of every set of paired stimuli in which we varied which stimulus sequence initiated stimulus broadcasts.

Testing apparatus

Phonotaxis experiments were conducted in an acoustically transparent circular arena (2-m diameter, 60-cm height) located inside a temperature-controlled hemi-anechoic sound chamber (length x width x height: 2.8 x 2.3 x 2.1 m; Industrial Acoustics Company, IAC, North Aurora, IL, USA). The inside walls and ceiling of the sound were covered in dark gray, perforated acoustic absorber panels and the floor was covered with dark gray carpet. The acoustically transparent arena wall was constructed from hardware mesh covered with black fabric. On the floor of the carpeted arena, just outside the arena wall, two speakers facing the center of the arena were positioned at an angular separation of 90°. A response zone consisting of a 10-cm semicircle was marked on the arena floor in front of each speaker. At the center of the arena floor a small, acoustically transparent release cage (9-cm diameter, 2-cm height) was located where subjects were placed at the beginning of each test. During tests, the arena was illuminated only with an overhead infrared (IR) light source (Tracksys Ltd, Nottingham, UK).

Stimuli were broadcast through two Mod1 Orb speakers (Orb Audio, New York, USA) using Adobe Audition 3.0 (Adobe Systems Inc., San Jose, CA, USA) interfaced with a MOTU model 16A sound card (MOTU, Inc., Cambridge, MA, USA), and Crown XLS 1000 High-Density Power Amplifiers (Crown International, Los Angeles, CA, USA). The frequency response of this system was flat (± 2.5 dB) within the frequency range of interest. At the start of each day, each stimulus to be presented from the two speakers was calibrated by placing a Type 4950 microphone connected to a Type 2250-L sound level meter (Bruël and Kjær, Nærum, Denmark) at the center of the arena floor (1 m from the speakers) such that it pointed directly towards the speaker to be calibrated. In four separate replicates of each test (Table 1), the overall playback level of each of the two paired stimuli was calibrated to an overall amplitude of either 85, 75, 65, or 55 dB SPL (re 20 µPa, fast, C-weighted).

Experimental design and protocol

We conducted two separate phonotaxis experiments to evaluate female preferences for the spectral content of conspecific advertisement calls. In Experiment 1 (see Fig. 1a), we performed a series of two-alternative choice tests to measure relative preferences for a bimodal call (1.25 kHz *and* 2.5 kHz) versus a unimodal call (either 1.25 kHz *or* 2.5 kHz). We replicated both of these choice tests at 12 factorial combinations of four overall amplitudes (55 dB, 65 dB, 75 dB, and 85 dB SPL) and three relative amplitudes between the two spectral components of the bimodal call (0 dB, -5 dB, and -11 dB). The nominal amplitudes of all stimuli are listed in Table 1. Note that because *overall* amplitude was held constant for both the bimodal and unimodal stimuli in a choice test, the *relative* amplitudes of 0 dB, -5 dB, and -11 dB instantiated between the two components within the bimodal call created *realized* amplitude differences of approximately -3.0 dB, -1.2 dB, and -0.3 dB, respectively, for the high-frequency component of the bimodal call relative to the high-frequency unimodal call (Table 1). Each subject's (n = 47) preference was measured in both choice tests (bimodal vs. low-frequency unimodal and bimodal vs. high-frequency unimodal), but not across all 12 factorial combinations of overall and relative amplitudes. One group of subjects (n = 25) experienced both choice tests at relative amplitudes of -11 dB and -5 dB, and a second group of subjects (n = 22) experienced both choice tests at the relative amplitude of 0 dB. The order in which the replicates of the two choice tests were presented was randomized for each subject. In Experiment 2 (see Fig. 2a), we performed a second series of two-alternative choice tests to measure preferences for low-frequency versus high-frequency unimodal calls across a range of overall sound amplitudes. We gave subjects (n = 28) a choice between two unimodal calls that differed only in their carrier frequency (1.25 kHz vs. 2.5 kHz; see Fig. 2a) at each of the four overall amplitudes (55 dB, 65 dB, 75 dB, and 85 dB SPL) in random order. Most subjects completed this choice test at all four overall amplitudes.

For both experiments, our procedures for conducting two-alternative choice tests were similar to those reported in previous studies (Bee 2010; Tanner et al. 2017; Gupta and Bee 2020; LaBarbera et al. 2020). Briefly, at the start of each test, a subject was separated from her mate and placed in a small acoustically transparent cage at the center of the test arena floor.

The subject was allowed to acclimate in silence in the release cage for 60 s, after which we started broadcasting the two alternating stimuli. For every choice test, the stimulus sequence initiating the alternating broadcasts was randomly selected at the start of each day. After the first two repetitions of calls from each speaker, the lid of the release cage was lifted remotely via a rope and pulley system, and the subject was allowed to move freely in the arena. Movements of subjects were observed on a video monitor connected to an IR-sensitive camera (Panasonic WV-BP334; Panasonic Corporation of North America, Secaucus, NJ, USA) and responses were scored in real-time. We recorded a response if a subject entered the response zone in front of an active speaker within 5 minutes of its release (subjects typically responded in under 2 min). The choice was scored as binary based on which stimulus was chosen. A score of 'no response' was recorded when the subject met any of the following criteria: it did not exit the release cage within 3 min; its first physical contact with the arena wall was in the quadrant opposite that spanning the wall between the two speakers; or it did not enter a response zone within 5 min. Preference was measured as the proportion of subjects choosing one stimulus over the other. Tests in which subjects did not respond were excluded from consideration (47 of 620 tests), yielding final sample sizes in each two-alternative choice test of between $n = 16$ and $n = 28$, with most tests having sample sizes of $n \geq 20$.

Statistical analyses

Statistical analysis was conducted in R version 4.2.0 (R core team) and $\alpha = 0.05$ was used for hypothesis testing. For each choice test, we conducted a one-tailed binomial test to evaluate the null hypothesis that subjects would choose both stimuli in equal proportions (i.e., null proportion = 0.50). Following an earlier study by Bee (2010), the alternative hypotheses were that females would prefer a bimodal call over a low-frequency unimodal call (Experiment 1), a bimodal call over a high-frequency unimodal call (Experiment 1), and a high-frequency unimodal call over a low-frequency unimodal call (Experiment 2). We followed the Holm-Šidák procedure to correct for multiple comparisons in tests of each of these alternative hypotheses across combinations of overall level and relative level. Because only one response per subject was used for any given binomial test, every data point was independent, meeting the assumptions of the binomial test. To evaluate the effect of overall amplitude (Experiments 1 and 2) and relative amplitude (Experiment 1) on the proportions of subjects choosing one stimulus over the over, we fitted generalized estimating equations (GEE) with logit link functions and exchangeable correlation structures using the *geepack* package (Højsgaard et al. 2016). For Experiment 1, we assessed the effect of overall and relative amplitudes on the proportions of subjects choosing the bimodal over the unimodal call by fitting a GEE model that included the unimodal alternative to the bimodal call (i.e., the low-frequency unimodal call or the high-frequency unimodal call), overall amplitude (55, 65, 75, or 85 dB SPL), relative amplitude (0, -5, or -11 dB), and their two-way interactions as predictor variables. Because this model contained six different main effects, we fitted seven additional models that systematically excluded the interaction terms (see Supplementary Information). Using a model selection method based on quasi-likelihood information criterion (QIC), which is an analog of AIC used for GEE and other models that use a quasi-likelihood method (Pan 2001), we selected the most parsimonious model that best fit the data (see Supplementary Information). We analyzed the main effects of the selected model by comparing it with other models without each main effect as a predictor variable. For Experiment 2, we assessed the effect of overall amplitude on the proportion of subjects choosing one unimodal call over the other by comparing two GEE models with and without overall amplitude as a predictor variable. In all statistical models, subject ID was used for clustering to account for repeated tests within subjects.

Results

Experiment 1 – bimodal vs. unimodal

Across all overall amplitudes and relative amplitudes, the proportion of subjects (n = 16 to 21) choosing the bimodal call over a unimodal call ranged between 0.34 and 1.00, with the proportion favoring the bimodal call at or above 0.75 in most choice tests (Figs. 1c, 1d; Table 2). Out of the 12 factorial combinations of overall and relative amplitudes, the proportion of subjects choosing the bimodal call over the low-frequency unimodal call was greater than 0.5 and associated with a p-value less than $\alpha = 0.05$ in 10 of 12 combinations, all of which remained significantly different from chance expectation after correction for multiple comparisons (Table 2). Preferences were not observed when the overall amplitude was 55 dB SPL and the relative amplitude was -5 dB or -11 dB (Table 2). In contrast, the proportion of subjects choosing the bimodal call over the high-frequency unimodal call was greater than 0.5 and associated with a p-value less than $\alpha = 0.05$ in six of the 12 combinations of overall and relative amplitudes but remained significantly different from chance expectation at only one combination after correction for multiple comparisons (65 dB and -5 dB; Table 2). Consistent with this overall pattern of outcomes from binomial tests, the proportion of subjects choosing the bimodal call was significantly higher when the alternative was the low-frequency unimodal call compared with the high-frequency unimodal call ($\chi^2 = 19.60$, $p < 0.001$; Figs. 1c, 1d). Averaged across overall and relative amplitudes, subjects chose the bimodal call over the low-frequency unimodal call in 86.5% of tests (205 of 237), whereas they chose the bimodal call over the high-frequency unimodal call in 66.0% of tests (157 of 238).

The proportion of subjects choosing the bimodal call over a unimodal alternative did not differ significantly as a function of overall amplitude ($\chi^2 = 4.27$, $p = 0.230$) or relative amplitude ($\chi^2 = 0.35$, $p = 0.840$) after averaging responses across the two choice tests (bimodal vs. unimodal hi-frequency and bimodal vs. unimodal low frequency). However, there was a significant interaction between relative amplitude and which unimodal call (low frequency vs. high frequency) served as the alternative to the bimodal call ($\chi^2 = 8.23$, $p = 0.016$; Fig. 1e). Averaged over all four overall amplitudes, the proportion of subjects choosing the bimodal call increased as a function of increasing relative amplitude when the alternative was the low-frequency unimodal call (Fig. 1e, open symbols), but this same proportion decreased with increasing relative amplitude when the alternative was the high-frequency unimodal call (Fig. 1e, closed symbols). In addition, there was a significant interaction between overall amplitude and relative amplitude ($\chi^2 = 12.9$, $p = 0.045$; Fig. 1f). This outcome reflects the opposing trends observed at a relative amplitude of 0 dB compared with -5 dB and -11 dB. At relative amplitudes of -5 dB and -11 dB, the proportion of subjects choosing the bimodal call over the high-frequency unimodal call *increased* as a function of increasing overall amplitude (Fig. 1f, blue and orange symbols and lines), whereas at the relative amplitude of 0 dB, the proportion of subjects choosing the bimodal call *decreased* with increasing amplitude (Fig. 1f, black symbols and lines). This two-way interaction was likely driven by a spectrum-dependent influence on how overall amplitude impacted preferences for the bimodal call when the relative amplitude of its two components was 0 dB (cf. Figs. 1c, 1d, diamond symbols, dashed black lines, and gray-shaded areas). When the alternative was the low-frequency unimodal call (Fig. 1c), the proportion of subjects choosing the bimodal call (with 0 dB relative amplitude) was consistently high (≥ 0.90, Table 2) across all overall amplitudes tested. But when the alternative was the high-frequency unimodal call (Fig. 1d), the proportion of subjects choosing the bimodal call (with 0 dB relative amplitude) decreased monotonically from 0.79 at 55 dB to 0.38 at 85 dB (Table 2).

Experiment 2 – high vs. low frequency

Across four overall amplitudes ranging between 55 dB and 85 dB SPL, the proportion of subjects (*n* = 22 to 28) that chose the high-frequency unimodal call over the low-frequency unimodal call ranged between 0.80 and 0.89 (Fig. 2b; Table 3). The proportions of subjects choosing the high-frequency call were significantly higher than expected by chance at all overall amplitudes (Table 3). The proportion of subjects choosing the high-frequency call did not differ significantly as a function of overall amplitude (Fig. 2b; $\chi^2 = 1.18$, $p = 0.758$). Averaged across all overall amplitudes, subjects chose the high-frequency call over the low-frequency call in 84.7% of tests (83 of 98).

Discussion

The main results of this study of female preferences for the spectral content of male advertisement calls can be summarized as follows. First, at most the combinations of overall and relative amplitude tested, more females chose calls with the species typical bimodal spectrum (i.e., 1.25 kHz *and* 2.5 kHz) over calls having just one spectral component (i.e., 1.25 kHz *or* 2.5 kHz). Second, preference for a bimodal spectrum was more pronounced when the alternative unimodal call consisted of the low-frequency spectral component. For example, at an overall amplitude of 65 dB SPL and higher, there was a significant preference for a bimodal call over a unimodal low-frequency call at all relative amplitudes tested. When the alternative was the unimodal high-frequency call, however, preferences for the bimodal call were more variable and dependent on relative amplitude, and a significant preference was found only at an overall amplitude of 65 dB SPL and relative amplitude of -5 dB. At no combination of spectral content, overall amplitude, and relative amplitude did females have a significant preference for a unimodal call over the bimodal alternative. Third, preferences for bimodal calls *increased*, albeit modestly, as a function of overall amplitude at most of the relative amplitudes tested for both unimodal alternative calls with one notable exception: when the unimodal call had only the high-frequency spectral component, preferences for the bimodal call *decreased* as a function of increasing overall level when its two spectral components had the same relative amplitude (i.e., relative amplitude of 0 dB). Finally, females exhibited significant and level-independent preferences for unimodal calls having just the high-frequency spectral component over one having just the low-frequency spectral component.

There is increasing awareness of the value of replicated studies in behavioral biology and related disciplines (Palmer 2000; Kelly 2006, 2019; Fraser et al. 2020). The present study of the Western clade of *H. chrysoscelis* was a “partial replication” (sensu Kelly 2006), as well as an extension, of an earlier study of the same clade by Bee (2010). That study used two-alternative choice tests conducted at a single overall amplitude (75 dB SPL) to examine female preferences for a bimodal call (1.1 kHz [-6 dB] *and* 2.2 kHz [0 dB) versus unimodal calls (1.1 kHz *or* 2.2 kHz) as well as preferences for a high-frequency (2.2 kHz) unimodal call versus a low-frequency (1.1 kHz) unimodal call. Females unanimously chose the bimodal call over the low-frequency unimodal alternative (1.1 kHz), but a somewhat lower proportion of females (67%) chose the bimodal call over the high-frequency unimodal alternative. When the amplitude of the unimodal alternative was adjusted so that it was equivalent to that of the same spectral component in the bimodal call (instead of the same overall amplitude of 75 dB SPL), females preferred the bimodal call over both unimodal alternatives, and again, the preference for bimodal calls over a unimodal alternative was somewhat greater when the alternative was the low-frequency unimodal call. In separate tests, females unanimously chose the high-frequency unimodal call over a low-frequency unimodal alternative. The overall pattern of results reported in the present study corroborates those reported in the earlier study by Bee (2010): females preferred bimodal calls over unimodal calls; this preference was more pronounced when the alternative was a low-frequency unimodal call; and females preferred high-frequency unimodal calls over low-frequency unimodal calls. The present study extends the earlier one by generalizing these broad patterns of preference over a 30-dB range of overall amplitudes and an 11-dB range of relative amplitudes in the bimodal call. Across most conditions, sound amplitude had modest or negligible impacts on female choice: preferences for the bimodal call over a unimodal alternative were similar or *increased* nominally as overall amplitude increased and they were also broadly similar across relative amplitudes, and preferences for a unimodal high-frequency call over a unimodal low-frequency call were independent of overall amplitude.

The combination of conditions tested in the present study that diverged from the overall pattern described above is when the two components in the bimodal call had a relative amplitude of 0 dB and the alternative was the high-frequency unimodal call. In these conditions, the proportion of subjects choosing the bimodal call *declined* with increasing overall amplitude, from 0.79 at 55 dB SPL to 0.38 at 85 dB SPL. The opposite trend was observed at relative amplitudes of -5 dB and -11 dB (Fig. 1d). We believe a consideration of several factors together potentially explain this divergent pattern. First, because overall amplitude was held constant across both alternatives in a choice test, *relative* amplitudes of 0 dB, -5 dB, and -11 dB in the bimodal call created *realized* amplitude differences of approximately 3.0 dB, 1.2 dB, and 0.3 dB, respectively, favoring the high-frequency unimodal call over the high-frequency component of the bimodal call (Table 1). Thus, at an overall amplitude of, say, 65 dB SPL, the amplitude of the high-frequency spectral peak was 65 dB in the unimodal call and approximately 62.0 dB, 63.8 dB, and 64.7 dB in the bimodal call at relative amplitudes of 0 dB, -5 dB, and -11 dB, respectively (Table 1). Second, previous studies of frogs (Fellers 1979; Arak 1983; Gerhardt 1987), including the Western clade of Cope's gray treefrog (Bee et al. 2012), suggest females probably do not behaviorally discriminate differences in amplitude much less than about 2 dB.

Third, at high overall amplitudes (e.g., 85 dB SPL), the low-frequency spectral peak of a bimodal call can have marked influences on preferences for bimodal calls over unimodal high-frequency calls even at very low relative amplitudes (e.g., -30 dB; Gerhardt 2005). However, at lower overall amplitudes (e.g., 55 dB SPL), the realized amplitude of the low-frequency spectral component at relative amplitudes of -5 dB and -11 dB is probably at or below the threshold sound level required to elicit a behavioral response from a large proportion of females (Nityananda and Bee 2012). Finally, given overlap in the tuning of auditory nerve fibers originating in the amphibian and basilar papillae at high sound amplitudes (e.g., Feng et al. 1975), a single high-frequency spectral component presented at high overall amplitudes (e.g., 85 dB SPL) would stimulate not only the basilar papilla, as it also would at lower overall amplitudes, but also the amphibian papilla, which is tuned to lower frequencies (Gerhardt and Schul 1999; Gerhardt 2005; Schrode et al. 2014). Together, these previous findings suggest the following interpretation of the present results. At the low (-11 dB) and intermediate (-5 dB) relative amplitudes, the realized amplitude differences between the high-frequency peaks in the bimodal and unimodal calls were less than 2 dB and thus unlikely to be behaviorally salient (Fellers 1979; Arak 1983; Gerhardt 1987; Bee et al. 2012). Consequently, the modest increase in preferences for the bimodal call observed as a function increasing overall level was likely driven by an increase in the behavioral salience of bimodal calls due to greater stimulation of the amphibian papillae by the low-frequency spectral component as overall level increased (Gerhardt and Schul 1999; Gerhardt 2005). In contrast, at the highest relative amplitude (0 dB), the realized amplitude difference between the high-frequency peaks in the bimodal and unimodal calls was 3 dB in favor of the unimodal call. Thus, we might have expected a slight preference for unimodal over bimodal calls if preferences were determined solely by differences in the realized amplitudes of the preferred high-frequency spectral component. Instead, females preferred the bimodal call at lower overall amplitudes (e.g., 55 dB SPL and 65 dB SPL; Fig. 1d) but not higher overall amplitudes (e.g., 75 dB SPL and 85 dB SPL; Fig. 1d). At lower overall amplitudes, the expected advantage of the high-frequency unimodal call was presumably offset due to sufficient stimulation of the amphibian papillae by the low-frequency spectral component at a relative amplitude of 0 dB. We hypothesize that the relative influence of the low-frequency spectral peak of the bimodal call on female responses was diminished at higher overall amplitudes (75 dB SPL and 85 dB SPL) because the high-frequency unimodal call by itself sufficiently stimulated both sensory papillae in the inner ear to offset any bimodal advantage (Gerhardt and Schul 1999; Gerhardt 2005; Schrode et al. 2014). These considerations are important because they highlight the number of factors that potentially impact the attractiveness of bimodal calls and hence patterns of intraspecific mate choice in natural populations where the relative amplitude of the low-frequency spectral peak can range from -1.7 dB to -24.2 dB (Ward et al. 2013).

The present study extends earlier work by Gerhardt and his colleagues (Gerhardt 1981a; b, 2005; Gerhardt and Doherty 1988; Gerhardt et al. 2007) investigating the role of spectral content in advertisement call recognition and female preferences in North American hylids. The subject of the present study, *H. chrysoscelis* (Cope's gray treefrog), is the diploid member of a cryptic diploid-tetraploid species complex in which *H. versicolor* (eastern gray treefrog) is the tetraploid. Extant populations of *H. chrysoscelis* can be divided into two geographically and genetically distinct lineages, a Western clade and an Eastern + Central clade, with the monophyletic Eastern clade nested within the Central lineage (Ptacek et al. 1994; Holloway et al. 2006; Booker et al. 2022). The Western clade extends from southern Canada to the U.S. Gulf Coast along the eastern boarder of the Great Plains. The Eastern + Central clade is found throughout the southern Midwest, the South, and along the southern East Coast of the U.S. (see maps in Booker et al. 2022). Genetic evidence suggests *H. versicolor* first arose through a single autopolyploid whole-genome duplication event within a now-extinct Northeastern clade of *H. chrysoscelis*, and that distinct extant lineages of *H. versicolor* have resulted from repeated hybridization with extant and extinct lineages of the diploid *H. chrysoscelis* (Bogart et al. 2020; Booker et al. 2022). The bird-voiced treefrog, *Hyla avivoca*, is the closely related sister species to the gray treefrog complex, and together with *H. chrysoscelis* and *H. versicolor* compose the *H. versicolor* species group (Faivovich et al. 2005; Hua et al. 2009).

Males in all lineages of *H. chrysoscelis* and *H. versicolor* produce calls with bimodal spectra, as do males of *Hyla arenicolor* (the canyon treefrog), a member of the closely related *Hyla eximia* species group (Gerhardt et al. 2007). In contrast, males of *H. avivoca* (in the *H. versicolor* group) produce an advertisement call with a unimodal spectrum consisting of a high-frequency spectral component (Gerhardt et al. 2007). Behavioral experiments with these species provide broad general support for a matched filter hypothesis, but also indicate considerable evolutionary lability in receiver mechanisms for processing spectral content (Gerhardt et al. 2007). As illustrated in Figure 3, females in species that produce advertisement calls with bimodal spectra (*H. chrysoscelis*, *H. versicolor*, and *H arenicolor*) prefer bimodal calls over at least one or both unimodal alternatives (high-frequency or low-frequency), whereas in the single species that produces a unimodal high-frequency call (*H. avivoca*), females prefer calls with this spectrum versus a bimodal alternative but reject unimodal low-frequency calls in favor of a bimodal call that includes the high-frequency spectral component (Fig. 3). Similar female preferences for bimodal over unimodal calls, at least at high overall amplitudes, have also been reported for both species of the closely related *Hyla cinerea* species group (*Hyla cinerea* and *Hyla gratiosa*), in which males also produce advertisement calls with bimodal spectra (Gerhardt 1981a, b; Lee et al. 2017). Together, these studies suggest a prominent role for parallel co-evolution between signals and receiver mechanisms when it comes to processing spectral content.

However, comparisons between cryptic sister species and between different genetic clades within a species also reveal that signal structure and the mechanisms underlying signal recognition and preferences are not always constrained by co-evolutionary processes (Schul and Bush 2002; Gerhardt et al. 2007). This is evident in comparing our results to those of Gerhardt et al. (2007). In females of the Western clade of *H. chrysoscelis* from Minnesota (this study), a bimodal call was strongly preferred over a low-frequency unimodal call across nearly all overall and relative amplitudes (Figs. 1c, 3a). In contrast, preferences for a bimodal call over a high-frequency unimodal call were weaker and more variable across overall and relative amplitudes (Figs. 1d, 3a). These data suggest the high-frequency spectral component has greater behavioral salience for females from the Western clade populations in Minnesota. Consistent with this interpretation was the robust and amplitude-independent female preferences observed for a high-frequency unimodal call over a low-frequency alternative (Figs. 2, 3b). These findings from the Western clade of *H. chrysoscelis* reveal stark differences in spectral preferences compared with *H. chrysoscelis* females from the Central and Eastern clades (Fig. 3; Gerhardt et al. 2007). In females from the Central clade in Missouri, bimodal calls were preferred over a high-frequency unimodal call to a greater extent than they were preferred over a low-frequency unimodal call (i.e., the opposite trend from Western-clade females; Fig. 3a). Moreover, Central-clade females from Missouri, and Eastern-clade females from North Carolina, were more likely to choose a low-frequency unimodal call paired against a high-frequency unimodal call (i.e., the opposite trend from Western-clade females; Fig. 3b). Likewise, in *H. versicolor*, Gerhardt et al. (2007) reported that females of both the Northwestern and Eastern lineages preferred bimodal calls over unimodal alternatives (Fig. 3a); however, females of the Northwestern lineage preferred high-frequency over low-frequency unimodal calls (as did females from a Southwestern lineage) while females of the Eastern lineage showed no preference (Fig. 3b). Thus, while females of all lineages of both *H. chrysoscelis* and *H. versicolor* tested to date prefer bimodal calls over a unimodal alternative, there is marked diversity across lineages in the relative behavioral salience of the high-frequency and low-frequency spectral components. At present, the mechanistic differences in spectral coding across lineages that underlies differences in how behavioral salience is assigned to different spectral peaks remains completely unknown.

In the first chapter of their co-edited volume on *Hearing and Sound Communication in Amphibians,* Narins and Feng (2007; p. 7) posed the following question: “The matched filter hypothesis—alive or dead?” The best answer that can be offered up from studies of spectral preferences in female treefrogs, albeit one that is somewhat of a cliché, is that “it’s complicated.” At a gross level, hylid treefrogs provide support for a role of matched filtering in call recognition. Across species, spectral preferences for bimodal over unimodal calls map directly onto species differences in the spectral content of advertisement calls (Gerhardt et al. 2007; Bee 2010; this study), suggesting a history of parallel co-evolution between signal structure and receiver mechanisms, as expected under a matched filter hypothesis. However, the observed diversity in spectral preferences within a cryptic species complex serves to highlight that even in taxonomic groups where signal structure is generally conserved and there is evidence of parallel co-evolution between signal structure and signal processing, there remains considerable potential for divergence in receiver mechanisms among closely related lineages and species, and potentially within lineages as well. The matched filter hypothesis would seem to be a poor explanation for intraspecific variation and intra-species-complex variation in spectral preferences, which instead speak to evolutionary lability in spectral preferences. The species and lineage differences in preferences for spectral content reported here and by Gerhardt et al. (2007) also build on other reports of within-species and between-species differences in the mechanisms of pulse rate processing and preferences for call duration and call rate in *H. chrysoscelis* and *H. versicolor* (Gerhardt 1994b; Schul and Bush 2002; Ward et al. 2013), as well as across-lineage but within-species differences in female preferences for spectral cues related to body-size in *H. chrysoscelis* (Morris 1989; Morris and Yoon 1989; Schrode et al. 2012). The unique evolutionary history of the *H. versicolor* species group, combined with its tractability for detailed quantitative behavioral, anatomical, biomechanical, and neurophysiological studies, makes this cryptic species complex an attractive one for future comparative neuroethological studies that aim to jointly test proximate and ultimate hypotheses about sound pattern recognition. Future studies in these systems, particularly those on spectral processing, could have no better guide in the literature for how to conduct robust experiments on sound pattern recognition than the groundbreaking contributions of Albert Feng.

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Declarations

Conflicts of interest The authors declare no competing or financial interests.

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Figure Legends

Fig. 1 Acoustic stimuli and results of Experiment 1. **a, b** Spectrograms illustrating a synthetic bimodal advertisement call paired against a low-frequency and a high-frequency unimodal call, respectively. **c** Proportion of subjects choosing the bimodal call as a function of overall and relative amplitudes when the alternative stimulus was the low-frequency unimodal call. **d** Proportion of subjects choosing the bimodal call as a function of overall and relative amplitudes when the alternative stimulus was the high-frequency unimodal call. **e** Proportion of subjects choosing the bimodal call, averaged across overall amplitudes, as a function of relative amplitude and the spectral component present in the unimodal alternative. **f** Proportion of subjects choosing the bimodal call, averaged across tests with the two unimodal calls, as functions of overall amplitude and relative amplitude. Error bars depict exact 95% binomial confidence intervals (CIs). Colored lines and shaded areas around the lines indicate the least squares fit lines and 95% CIs, respectively, from GEE analyses. A horizontal dashed line indicates the expected null proportion of 0.50 in a two-alternative choice test. Fractions show the number of subjects choosing the bimodal call (numerator) and the number of subjects tested (denominator).

Fig. 2 Acoustic stimuli and results for Experiment 2. **a** Spectrogram illustrating the spectral content of the two synthetic unimodal calls having a single carrier frequency of either 1.25 kHz or 2.5 kHz. **b** Proportions of subjects choosing the high-frequency unimodal call over the low-frequency unimodal call as a function of overall amplitude. Error bars depict exact 95% binomial confidence intervals (CIs). The sold black line and the shaded gray area around the line indicate the least squares fit line and 95% CI, respectively, from a GEE analysis. A horizontal dashed line illustrates the expected null proportion of 0.50 in a two-alternative choice test. Fractions show the number of subjects choosing the high-frequency unimodal call (numerator) and the number of subjects tested (denominator).

Fig. 3 Female preferences for spectral content across four species of *Hyla*. **a** Percentages of females choosing a bimodal call over either a high-frequency unimodal call (closed symbols) or a low-frequency unimodal call (open symbols). Dotted lines connect tests conducted within the same genetically distinct clade. **b** Percentages of females choosing a high-frequency unimodal call over a low-frequency unimodal call. Data from this study of *H. chrysoscelis* (square symbols) are taken from tests conducted at an overall amplitude of 85 dB SPL (in **a** and **b**) and at a species-typical relative amplitude of -11 dB (in **a**). Data for other clades and species are redrawn from Gerhardt et al. (2007) and represent data from tests conducted at or close to an overall amplitude 85 dB SPL and with species-typical relative amplitudes (except for *H. avivoca*, which does not produce a bimodal call). A stylized spectrum under each species' name depicts the natural spectral content of its advertisement calls. For *H. chrysoscelis*, the separate genetic clades indicated are the Western (W), Central (C), and Eastern clades following Booker et al. (2022). For *H. versicolor*, different Eastern (E), Northwestern (NW), and Southwestern (SW) clade designations follow Gerhardt et al. (2007). Error bars depict exact 95% binomial confidence intervals. Asterisks indicate significant preferences ($p < 0.05$) in two-tailed binomial tests (to facilitate comparison between studies) and horizontal dashed bars indicate the null expectation of 50% in a two-alternative choice test. See Gerhardt et al. (2007) for additional details.

Table 1 Nominal amplitudes of unimodal calls (both high-frequency and low-frequency calls) and each spectral peak of the bimodal call as functions of the experimentally determined relative amplitude and overall amplitude of each stimulus.

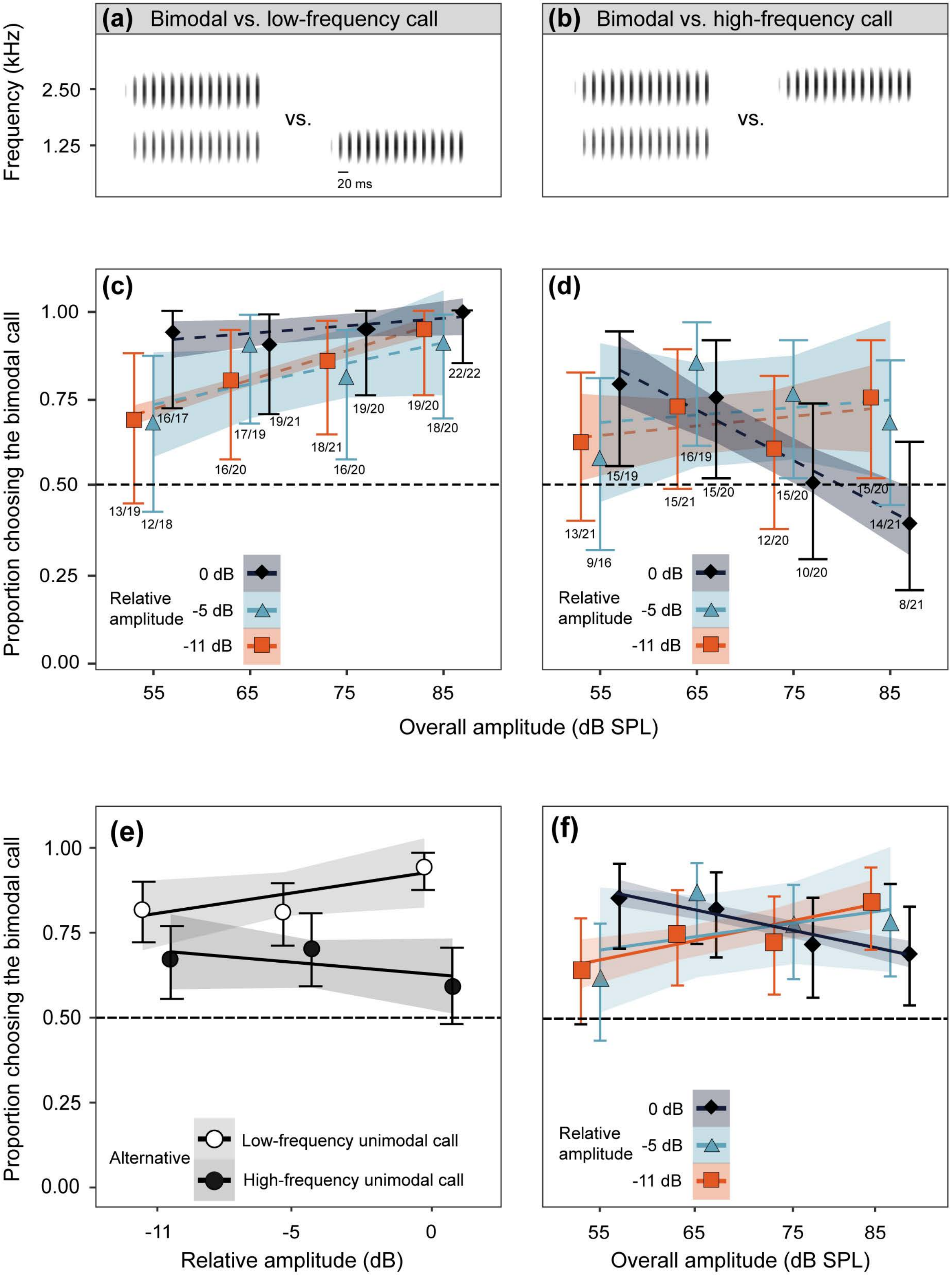
			Bimodal call	
Relative amplitude (dB)	Overall amplitude (dB SPL)	Unimodal call (dB SPL)	High-frequency component (dB SPL)	Low-frequency component (dB SPL)
0	55	55.0	52.0	52.0
	65	65.0	62.0	62.0
	75	75.0	72.0	72.0
	85	85.0	82.0	82.0
-5	55	55.0	53.8	48.8
	65	65.0	63.8	58.8
	75	75.0	73.8	68.8
	85	85.0	83.8	78.8
-11	55	55.0	54.7	43.7
	65	65.0	64.7	53.7
	75	75.0	74.7	63.7
	85	85.0	84.7	73.7

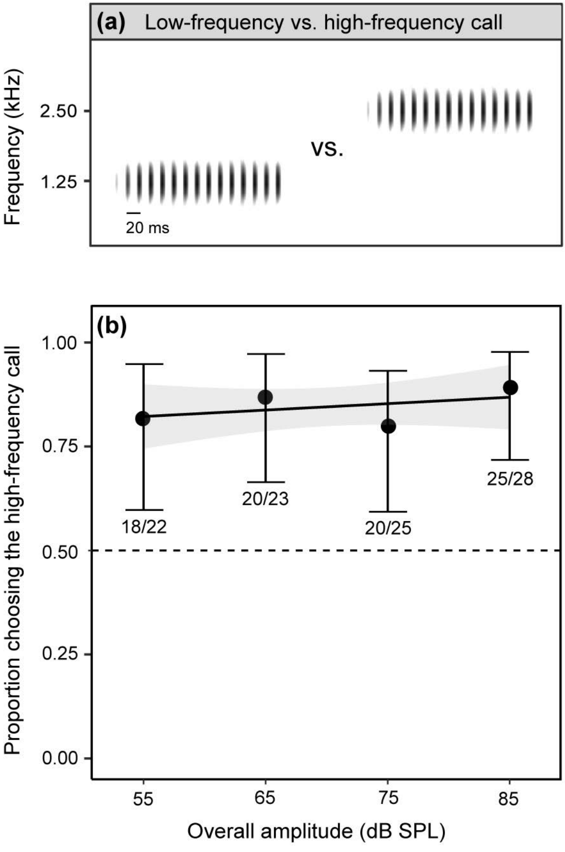
Table 2 Results of two-alternative choice tests from Experiment 1 indicating the sample sizes (*n*) and proportions of subjects choosing the bimodal call over the low-frequency and high-frequency unimodal calls in tests conducted at different combinations of overall amplitude (dB SPL) and relative amplitude (dB). Also shown are the 95% exact binomial confidence intervals (CI) around the observed proportions and the outcomes (*p* values) of one-tailed binomial tests (null proportion = 0.50). *P*-values less than $\alpha = 0.05$ are highlighted in **bold text**, and significant outcomes following correction for multiple comparisons are indicated with an asterisk (*).

Choice Test	Overall amplitude	Relative amplitude	*n*	Proportion	95% CI	*p*
Bimodal vs low-frequency unimodal	55 dB	0 dB	17	0.94	0.71 – 1.00	**< 0.001***
		-5 dB	18	0.67	0.41 – 0.87	0.119
		-11 dB	19	0.68	0.43 – 0.87	0.084
	65 dB	0 dB	21	0.90	0.70 – 0.99	**< 0.001***
		-5 dB	19	0.89	0.67 – 0.99	**< 0.001***
		-11 dB	20	0.80	0.56 – 0.94	**0.006***
	75 dB	0 dB	20	0.95	0.75 – 1.00	**< 0.001***
		-5 dB	20	0.80	0.56 – 0.94	**0.006***
		-11 dB	21	0.86	0.63 – 0.97	**0.001***
	85 dB	0 dB	22	1.00	0.85 – 1.00	**< 0.001***
		-5 dB	20	0.90	0.68 – 0.99	**< 0.001***
		-11 dB	20	0.95	0.75 – 1.00	**< 0.001***
Bimodal vs high-frequency unimodal	55 dB	0 dB	19	0.79	0.54 – 0.94	**0.010**
		-5 dB	16	0.56	0.30 – 0.80	0.402
		-11 dB	21	0.62	0.38 – 082	0.192
	65 dB	0 dB	20	0.75	0.51 – 0.91	**0.021**
		-5 dB	19	0.84	0.60 – 0.97	**0.002***
		-11 dB	21	0.71	0.48 – 0.89	**0.039**
	75 dB	0 dB	20	0.50	0.27 – 0.73	0.588
		-5 dB	20	0.75	0.50– 0.91	**0.021**
		-11 dB	20	0.60	0.36 – 0.81	0.252
	85 dB	0 dB	21	0.38	0.18 – 0.62	0.192
		-5 dB	21	0.67	0.43 – 0.85	0.095
		-11 dB	20	0.75	0.51 – 0.91	**0.021**

Table 3 Results of two-alternative choice tests from Experiment 2 indicating the sample sizes (*n*) and the proportions of subjects choosing the high-frequency unimodal call over the low-frequency unimodal call in tests conducted at different overall amplitudes (dB SPL). Also shown are the 95% exact binomial confidence intervals (CI) around the observed proportions and the outcomes (*p* values) of one-tailed binomial tests (null proportion = 0.50). *P*-values less than α = 0.05 are highlighted in **bold text**, and significant outcomes following correction for multiple comparisons are indicated with an asterisk (*).

Overall amplitude	*n*	Proportion	95% CI	*p*
55 dB	22	0.82	0.60 – 0.95	**0.002***
65 dB	23	0.87	0.66 – 0.97	**< 0.001***
75 dB	25	0.80	0.59 – 0.93	**0.002***
85 dB	28	0.89	0.72 – 0.98	**< 0.001***


(a) Bimodal vs. low-frequency call
(b) Bimodal vs. high-frequency call
Frequency (kHz)
2.50
1.25
vs.
20 ms
vs.
(c)
(d)
Proportion choosing the bimodal call
1.00
0.75
0.50
0.25
0.00
16/17
19/21
19/20
19/20
22/22
17/19
18/20
16/20
18/21
16/20
13/19
12/18
15/19
16/19
15/20
15/21
15/20
15/20
14/21
13/21
9/16
12/20
10/20
8/21
0 dB
Relative amplitude
-5 dB
-11 dB
55
65
75
85
Overall amplitude (dB SPL)
(e)
(f)
Alternative
Low-frequency unimodal call
High-frequency unimodal call
-11
-5
0
Relative amplitude (dB)
Overall amplitude (dB SPL)


(a) Low-frequency vs. high-frequency call
Frequency (kHz)
2.50
1.25
vs.
20 ms
(b)
Proportion choosing the high-frequency call
1.00
0.75
0.50
0.25
0.00
18/22
20/23
20/25
25/28
55
65
75
85
Overall amplitude (dB SPL)


(a)
Females choosing bimodal call (%)
100
90
80
70
60
50
40
30
20
10
0
*
bimodal preferred
unimodal preferred
W
W
C
C
H. chrysoscelis
E
NW
NW
H. versicolor
H. avivoca
H. arenicolor
(b)
Females choosing high-frequency unimodal call (%)
high-frequency preferred
low-frequency preferred
W
C-MO
E-NC
H. chrysoscelis
E
NW
SW
H. versicolor
H. avivoca
H. arenicolor

Supplementary Information

Female preferences for the spectral content of advertisement calls in Cope's gray treefrog (*Hyla chrysoscelis*)

Saumya Gupta and Mark A. Bee

Supplementary Table 1: Results of model selection from Experiment 1 indicating the quasi-likelihood values, quasi-likelihood information criterion (QIC) values, delta, and QIC weights. The model highlighted in bold had the lowest QIC value. Across models the predictor variables considered included the unimodal alternative to the bimodal call (i.e., the low-frequency unimodal call or the high-frequency unimodal call), overall amplitude (55, 65, 75, or 85 dB SPL), relative amplitude (0, -5, or -11 dB), and their two-way interactions.

Model	**Quasi-likelihood**	**QIC**	**Delta**	**Weight**
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:overall amplitude + unimodal alternative:relative amplitude + overall amplitude:relative amplitude	-228.969	495.6	2.03	0.168
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:overall amplitude + unimodal alternative:relative amplitude	-233.936	494.9	1.30	0.242
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:overall amplitude + overall amplitude:relative amplitude	-232.996	498.5	4.89	0.040
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:relative amplitude + overall amplitude:relative amplitude	**-232.588**	**493.6**	**0.00**	**0.464**
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:overall amplitude	-239.131	501.4	7.81	0.009
unimodal alternative + overall amplitude + relative amplitude + unimodal alternative:relative amplitude	-239.011	497.4	3.78	0.070
unimodal alternative + overall amplitude + relative amplitude + overall amplitude:relative amplitude	-237.995	502.9	9.25	0.005
unimodal alternative + overall amplitude + relative amplitude	-244.182	504.7	11.11	0.002