The Journal of Experimental Biology 210, 2990-2998
Published by The Company of Biologists 2007
doi:10.1242/jeb.006312

Preferences based on spectral differences in acoustic signals in four species of treefrogs (Anura: Hylidae)

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Accepted 26 June 2007

Summary

Frogs have two inner ear organs, each tuned to a different range of frequencies. Female treefrogs (Hylidae) of three species in which males produce calls with a bimodal spectrum (*Hyla chrysoscelis*, *H. versicolor*, *H. arenicolor*) preferred alternatives with a bimodal spectrum to alternatives with a single high-frequency peak. By contrast, females of *H. avivoca*, in which males produce calls with a single, high-frequency peak, preferred synthetic calls with a single high-frequency peak to calls with a bimodal spectrum. These results are consistent with the expectations of the matched-filter hypothesis and run counter to the predictions of the pre-existing bias hypothesis. At moderate to high playback levels (85–90 dB), females of *H. avivoca* and of two of three mtDNA-defined lineages of *H. versicolor* preferred unimodal signals with a high-frequency peak to those with a low-frequency peak. Females of *H. chrysoscelis*, *H. arenicolor* and the third lineage of *H. versicolor* did not show a preference, indicating that receiver mechanisms may be at least as evolutionarily labile as call structure. Spectral-peak preferences of gray treefrogs from Missouri, USA were intensity-dependent. Whereas females chose low-frequency calls at 65 dB SPL, there was either no preference (*H. chrysoscelis*) or a preference for high-frequency calls (*H. versicolor*) at 85 and 90 dB SPL. These non-linear effects indicate that there is an increasing influence of high-frequency energy on preferences as females approach calling males, and these results serve to emphasize that playback experiments conducted at a single level may have limited generality.

Key words: acoustic communication, matched filtering, pre-existing bias, non-linear effect, *Hyla*, spectral-structure preference.

Introduction

Animals with sensory specializations have been favorite subjects for research in both evolutionary biology and neurobiology. The advantage for evolutionary studies is that intraspecific communication may then depend on a relatively circumscribed set of signal properties of a single sensory modality. Research concerned with mate choice, for example, can thus focus on variation in signals and preferences that are necessarily under strong selective pressure. For the neurobiologist, sensory specializations facilitate the search for functionally important neurons and networks that are likely to be over-represented in the sensory system (e.g. over-representation of echo-location call frequencies in the peripheral auditory system of bats, interaural time-sensitive neurons in barn owls, and texture-sensitive tactile neurons in star-nosed moles) (Koppl et al., 1993; Catania and Henry, 2006; Suga, 1979). In this study, the peripheral specialization of the auditory system of frogs sets up a straightforward test of broad interpretations of the matched-filter and pre-existing bias hypotheses.

Frogs and toads have served as important model systems for the study of both evolution and mechanisms of auditory processing because of their strong reliance on acoustic communication (for a review, see Gerhardt and Huber, 2002). Their auditory system is specialized at the peripheral level by virtue of having two inner ear organs with different frequency sensitivities and physiological properties (for a review, see Feng and Schellart, 1999). Capranica (Capranica, 1965; Capranica and Moffat, 1983) pointed out the correspondence of the tuning of these two organs with the frequency bands emphasized in the conspecific advertisement call of several species of anurans and popularized the matched-filter hypothesis. Under this hypothesis, the auditory system is maximally sensitive to frequencies strongly represented in conspecific calls and less sensitive to (filters out) the signals of other species and abiotic noise, which are expected to differ in their spectral content from conspecific signals. A matched-filter system would therefore facilitate communication and signal recognition in noisy environments. Comparative studies have confirmed Capranica's expectation of auditory tuning to conspecific-call frequencies in a reasonably large sample of taxonomically diverse anurans, although many of the estimates were based on neurophysiological measurements from the central auditory system rather than directly from the auditory nerve (for a review, see Gerhardt and Schwartz, 2001).

Another expectation arising from Capranica's interpretation of the matched-filter hypothesis is that, if there are two spectral peaks in the advertisement call of a given species, then a signal with both peaks will be much more effective in intraspecific communication than a signal having only one of these peaks (Capranica, 1965). Support for this prediction was provided by studies of evoked calling in bullfrogs (*Rana catesbeiana*) (Capranica, 1965) and by preference tests in several North American treefrogs (for a review, see Gerhardt, 2001). Neural correlates of these behavioral results were found in a systematic survey of frequency selectivity in a subset of single neurons in the midbrain and thalamus of the leopard frog (*Rana pipiens*) (Fuzessery and Feng, 1983) (for a review, see Fuzessery, 1988) and in evoked potentials in the auditory thalamus of bullfrogs and green treefrogs (*Hyla cinerea*) (Mudry and Capranica, 1987a; Mudry and Capranica, 1987b). Neural activity was best elicited when acoustic stimuli had two widely separated frequency components corresponding to emphasized frequency bands in conspecific calls; stimulation by either component alone had little or no effect. A recent study of *Hyla versicolor* showed, however, that extensive lesions to the auditory thalamus did not abolish phonotaxis and had little or no effect on the usual preference for a bimodal stimulus (Endepols et al., 2003). The locus of the behaviorally relevant convergence of inputs from the two auditory ear organs thus either differs among species or, more likely, occurs lower in the auditory pathway, probably within the torus semicircularis (Fuzessery, 1988; Endepols et al., 2003; Hoke et al., 2004).

Although the matched-filter hypothesis is supported at a gross level of analysis, there are also many examples of mismatches between auditory tuning and the mean frequency or frequencies emphasized in conspecific calls (e.g. Ryan et al., 1992). These mismatches potentially result in sexual selection on the frequency content of male signals (for a review, see Gerhardt and Huber, 2002). More significantly, the existence of two inner ear organs and facilitated responses to combinations of frequencies in the central auditory system sets up the possibility for a rapid evolutionary change in the spectral properties of species in which the advertisement call has a single spectral peak. That is, if a mutation arises that results in calls with substantial energy in a second frequency matching the sensitivity of the second inner ear organ, such a call may be immediately favored by female mate choice. This kind of scenario is consistent with the pre-existing bias or sensory exploitation hypothesis (e.g. Ryan and Rand, 1990). In the context of the anuran auditory system, this hypothesis assumes that the outputs of both inner ear organs contribute positively to the activation of the central integrating system that ultimately drives selective phonotaxis. The contribution of the peripheral inputs could be approximately equal or might differentially enhance the activation of the central integrating system (see Discussion). Another possibility, which is incompatible with the pre-existing bias hypothesis in its broadest interpretation, is that the input from one inner organ could have an excitatory effect, and the input from the second organ, a suppressive effect. In this scenario, a bimodal stimulus would be less attractive than a stimulus with a single peak. Such a system could be considered a matched filter if the energy in conspecific signals matched the sensitivity of the inner ear organ with the excitatory effect. Furthermore, the original formulation of the matched-filter hypothesis assumed that the relative importance of low-frequency and high-frequency stimulation remains constant, but the relative stimulation by the two frequencies could change with changes in the absolute intensity of the advertisement call. A complete understanding of the underlying mechanisms must thus consider non-linear effects such as the intensity-dependence of facilitated responses (Gerhardt, 1981), which may be influenced by cross-talk between the two inner ear organs. Cross-talk, in turn, is expected to occur at the relatively high amplitudes (⩾80 dB SPL) (Gerhardt and Schul, 1999; Gerhardt, 2005a) experienced by female frogs as they approach calling males (Gerhardt and Klump, 1988).

We compared the preferences of four species of North American treefrogs (Anura: Hylidae) with respect to two characteristics of the spectral structure of their advertisement calls: (1) the number of spectral peaks (one *versus* two) and (2) the relative attractiveness of those two peaks. Three of these species are closely related and occur in the eastern third of the USA (the gray treefrogs *Hyla chrysoscelis* and *H. versicolor*, and the bird-voiced treefrog *H. avivoca*); the fourth species, the canyon treefrog (*H. arenicolor*) is currently considered to be in a sister clade, with its closest relatives mainly occurring in Mexico (Faivovitch et al., 2005), where it is also widely distributed. We also assessed the intensity-dependence of the relative attractiveness of the two peaks in both species of gray treefrogs from the Midwestern USA.

Our comparative approach had three specific goals. First, we tested the prediction of the matched-filter hypothesis that females of species in which conspecific calls have bimodal spectra (gray treefrogs, *H. arenicolor*) would prefer calls with both spectral peaks to either peak alone. Second, we tested the matched-filter and pre-existing bias hypotheses in the bird-voiced treefrog, a closely related species in which the advertisement call is a nearly pure-tone trill of relatively high frequency that is likely to be processed mainly by the basilar papilla. A preference for a call with a single high-frequency peak over a bimodal stimulus would be consistent with the matched-filter hypothesis because the preference corresponds to the spectral structure of conspecific calls. In contrast, the expectation of the pre-existing bias hypothesis is that adding a second, lower-frequency peak in the range of sensitivity of the amphibian papilla should result in a stimulus that is more attractive than the conspecific call (Ryan and Rand, 1990); see Discussion. Experiments of this kind have been conducted in two other anuran species with calls having a single spectral peak (Witte et al., 2001; Bosch and Boyero, 2003), but none of the relatives of these species produces calls with bimodal spectra like the closest relatives of *H. avivoca*, the two species of gray treefrogs, in which females also show preferences for calls with bimodal spectra (Gerhardt and Doherty, 1988; Gerhardt, 2005b) (present study). Third, we tested for interspecific and intraspecific differences in the relative attractiveness of single-peaked calls of either low (matching the tuning of the amphibian papilla) or high frequency (matching the tuning of the basilar papilla). Initial experiments (Gerhardt and Doherty, 1988) with *H. versicolor* involved females from only one of three lineages, as defined by mitochondrial DNA sequences (Ptacek et al., 1994). A recent study indicates that these lineages evolved as allopolyploids involving ancestral *H. chrysoscelis* and two other extinct taxa (Holloway et al., 2006). The calls of these lineages are similar, however, and there is evidence of between-lineage gene exchange (Espinoza and Noor, 2002; Holloway et al., 2006). Species and especially lineage differences in preferences in the absence of differences in spectral structure would support the idea that signals and receiver preferences are not necessarily tightly constrained by co-evolutionary processes (e.g. Ryan and Rand, 1990; Schul and Bush, 2002).

Materials and methods

Acoustic stimuli

The advertisement calls of *Hyla versicolor* Le Conte 1825, *Hyla chrysoscelis* Cope 1880 and *Hyla avivoca* Viosca 1928 have been described previously (Gerhardt, 1974; Gerhardt, 2001; Gerhardt, 2005a; Gerhardt, 2005b). Analyses of recordings of 17 males of *Hyla arenicolor* Cope 1866 from southeastern Arizona were used to characterize the acoustic properties of its advertisement call. The average values of the acoustic properties of these calls were used as guides for generating the synthetic calls used in the playback experiments.

Synthetic calls were made with custom-designed software (written by J. J. Schwartz) that created 8-bit or 16-bit digital files with an output sampling rate of at least 20 kHz. For laboratory playback experiments, the temporal properties of the synthetic calls were typical of calls produced by conspecific males calling at about 20°C, which was also the temperature (±2°C) at which females were tested in laboratory playbacks. One exception was the absence of within-pulse amplitude modulation (subpulses) that is typical of the calls of *H. arenicolor*. Including this feature results in a more complex spectral structure (the addition of sidebands around the two emphasized frequency bands) that would have precluded direct comparisons with the other species. Females responded readily to these simplified synthetic calls. Tests of *H. versicolor* in Virginia and of *H. avivoca* were conducted in the field at ambient temperatures of 17.8–22.8°C and 17–23°C, respectively. Females of *H. avivoca* were tested with synthetic-call alternatives in which the temporal properties matched those of typical males at 16°C, 20°C or 24°C; the stimuli used depended on the ambient temperature in the field at the time of the playback experiments. Call periods were fixed at species-typical values. The gross timing relationship of alternatives in any given test were fixed so that there were equal periods of silence between the end of one stimulus and the beginning of the other stimulus.

The frequencies of the spectral peaks of the synthetic calls were typical of those in conspecific calls except for the bimodal stimulus used to test females of *H. avivoca* (Table 1). We chose the frequency of the second peak of this stimulus to be one-half that of the high-frequency peak because the two spectral peaks in the calls of the gray treefrogs consist of two harmonically related components of somewhat lower frequency (Gerhardt, 2005b). We chose to adjust the relative amplitudes of two peaks in this stimulus to the same value so that our results could be compared directly with those of previous studies in which females of species having single-peaked calls were tested with bimodal stimuli (Witte et al., 2001; Bosch and Boyero, 2003). Even though the low-frequency peak in the calls of gray treefrogs typically has less energy than the high-frequency peak, females preferred bimodal stimuli to alternatives with a single high-frequency peak over a wide range of relative amplitudes, including 0 dB (H.C.G., unpublished data).

Table 1. *Spectral peaks in synthetic calls*

Species	Low-frequency peak (Hz)	High-frequency peak (Hz)	Relative amplitude (dB)
H. versicolor	1.1	2.2	6±2
H. chrysoscelis	1.2	2.4	6±2
H. avivoca	1.3*	2.6	0±2
H. arenicolor	0.65	2.6	0±2

*The conspecific advertisement call lacks energy at this frequency, which was chosen because the low-frequency peaks in the calls of the two closest relatives of this species are centered at about one-half of the peak frequency of the typically dominant band.

The bandwidths of spectral peaks were somewhat narrower in synthetic calls than in natural calls because synthetic calls did not incorporate the within-pulse frequency modulation typical of these species. In tests of *H. versicolor* and *H. chrysoscelis* these standard calls were as attractive as synthetic calls with frequency-modulated pulses which, in turn, were as attractive as those of typical calls recorded in the field (Gerhardt, 1978; Gerhardt, 2005b). In the advertisement calls of *H. avivoca* and *H. arenicolor*, the extent of within-pulse frequency modulation is much less than that in the calls of the gray treefrogs.

Playback systems

Females of *H. versicolor* from Virginia, USA (Table 2) were tested in a playback arena located 200 m from the ponds where the frogs were collected. The playback area was a level soil surface with low herbaceous growth trimmed to permit observation of females throughout their phonotactic approaches; females were viewed under dim illumination from a rheostat-controlled headlamp. Sounds were played back from two Acoustic Research Edge Sequel speakers separated by 2 m and amplified by a Kenwood KAC 600 amplifier (Kenwood, USA, Long Beach, CA, USA). Digital files were output from an Apple 5300ce laptop (Apple, Cupertino, CA, USA) using Soundedit 16 version 2. The amplitudes of alternative acoustic stimuli were equalized at 85 dB SPL [‘fast’ root-mean-square (RMS) meter setting] at the release point using a RadioShack sound level meter (Radio Shack, Fort Worth, TX, USA). Ambient background noise was <50 dB SPL (C-weighted, fast RMS).

Females of *H. avivoca* from Mississippi, Tennessee and South Carolina (Table 2) were tested outdoors in an arena (2 m×1 m), with 50 cm high wooden frames supporting a light black cloth that shielded the movements of the researcher and the positions of the loudspeakers from the frogs. The floor of the arena was a level cement slab. Tests were conducted at sites well away from the nearest chorus. Sounds, which were conditioned and amplified by custom-designed amplifiers and

Table 2. *Sources of females*

Species	Locality	Coordinates	Number collected	Number responding
Hyla chrysoscelis[1]	Phelps Co., MO	37°42′N, 91°59′W	134	122
		37°37′N, 92°08′W		
	Laclede Co., MO	37°53′N, 92°43′W		
	Texas Co., MO	37°29′N, 92°3′W	7	7
	Orange Co., NC	36°03′N, 79°06′W	27	21
		35°57′N, 79°04′W		
		35°54′N, 79°01′W		
Hyla versicolor[2]	Boone Co., MO	38°45′N, 92°12′W	202	166
	Phelps Co., MO	37°42′N, 91°59′W	4	4
	Augusta Co., VA	38°25′N, 79°08′W	13	12
	Westchester Co., NY	41°17′N, 73°55′W	33	30
	Bastrop Co., TX	30°07′N, 97°17′W	11	8
	Smith Co., TX	32°12′N, 95°10′W	1	1
	Anderson Co., TX	31°56′N, 95°52′W	5	5
Hyla avivoca[3]	Perry Co., MS	31°13′N, 89°10′W	24	23
		31°09′N, 88°55′W	15	14
	Lake Co., TN	36°24′N, 89°21′W	7	7
	Hampton Co., SC	32°34′N, 81°17′W	18	16
Hyla arenicolor[1]	Gila Co., AZ	33°51′N, 110°57′W	2	2
		33°50′N, 110°59′W	3	2
		33°48′N, 110°58′W	2	2
	Pima Co., AZ	31°44′N, 110°53′W	9	7
		31°48′N, 110°47′W	2	2
		31°46′N, 110°51′W	3	3
		32°20′N, 110°42′W	5	5
	Coconino Co., AZ	34°32′N, 111°17′W	2	0
	Santa Cruz Co., AZ	31°44′N, 110°42′W	3	3

[1] All females were tested in a semi-anechoic chamber in Missouri.

[2] Females from VA tested in the field >200 m from the collecting site. Females from MO and TX tested in a semi-anechoic chamber in Missouri. Females from NY tested in a semi-anechoic chamber in NY. Females from MO are representative of the NW mtDNA lineage; females from TX are representative of the SW mtDNA lineage; females from NY and VA are representative of the E mtDNA lineage (see definitions and map in Ptacek et al., 1994).

[3] Females were tested in the field >200 m from the collecting site.

AZ, Arizona; MO, Missouri; NC, North Carolina; NY, New York; TN, Tennessee; TX, Texas; VA, Virginia.

attenuators, were played back from two Analog-Digital Systems 200 speakers (Boston, MA, USA), which were separated by 2 m and placed just outside and at either end of the arena. Digital files were output from a McIntosh iBook (Cupertino, CA, USA) using Raven 1.2.1 (Cornell Laboratories 2003–2005). The amplitude of acoustic stimuli was adjusted to 85 dB SPL (C-weighted, fast RMS) at the release point using a CEL-254 Digital Impulse Sound Level Meter (Bedford, UK). Ambient background noise was <50 dB SPL (C-weighted, fast RMS).

Females of *H. versicolor* from New York (Table 2) were tested indoors in a basement room lined with anechoic foam wedges (TFW-4; Silent Source, Northampton, MA, USA) at Pace University (Pleasantville, NY, USA) and floored with waterproof, low-pile carpet. Sounds were amplified by a Realistic SA-10 amplifier and played back from two Realistic Minimus 0.3 speakers (RadioShack), which were separated by 2 m. Digital files were output from a Commodore Amiga 600 computer (Commodore Business Machines; West Chester, PA, USA) using custom-designed software. The amplitude of acoustic stimuli was equalized at 85 dB SPL (C-weighted, fast RMS) at the release point using a General Radio 1982 precision sound level meter (IET Labs; Westbury, NY, USA). Ambient background noise was <50 dB SPL (C-weighted, fast RMS).

Females of *H. versicolor* (Missouri and Texas, USA), *H. chrysoscelis* (Missouri and North Carolina, USA), and *H. arenicolor* (Arizona, USA) (Table 2) were tested in the semi-anechoic chamber as described (Gerhardt, 1994). Sounds were amplified by Nagra DSM amplifiers and played back from two Analog-Digital-Systems 200 speakers, which were separated by 2 m. Digital files were output from an IBM-compatible personal computer using CoolEdit Pro, Adobe Audition (Syntrillium, Adobe, Mountain View, CA, USA), or software specific to digital-to-analog interfaces (Siliconsoft supersound 8-bit board or Siliconsoft DacEditor 12AB; 16-bit, San Jose, CA, USA). Signal amplitudes were adjusted (alternatives equalized at 65 dB, 75 dB, 85 dB or 90 dB spl, C-weighted, fast RMS) using a General Radio 1990 (IET Labs; Westbury, NY, USA) or Larsen-Davis 720 (Provo, UT, USA) sound level meter. Ambient background noise was <50 dB SPL (C-weighted, fast RMS).

The frequency–response of all playback systems was sufficiently flat to ensure that the relative amplitudes of the two peaks in bimodal signals were within ±3 dB of the target values.

Because preliminary tests indicated that females of *H. avivoca* preferred a single-peaked call of 2.6 kHz to the bimodal alternative, we adjusted the SPL of the bimodal stimulus to be 3 dB greater than that of the single-peaked call (86 *vs* 83 dB SPL, respectively). This procedure ensured that the absolute values of the single-peaked (2.6 kHz) call and the 2.6 kHz-component of the bimodal stimulus were the same with a narrow margin of error. This assertion was checked by analysis of recordings and sound level measurements (Brüel & Kjaer 2209 sound level meter and 3% tunable filter) made at the release point. The relative amplitudes of the two spectral peaks of the bimodal stimulus were within ±1 dB. The absolute value of the single-peaked components was within ±1 dB of the value of the same component in the bimodal stimulus when the alternatives were equalized (±1 dB) at the release point. Error margins refer to the specifications of the sound level meter and tuneable filter and not the values that we observed, which were within ±0.3 dB of the target values.

There are several reasons to be confident that the results of the tests we report here are comparable despite the different testing setups. First, the results of the two tests reported here were the same for eastern-lineage females of *H. versicolor* from Virginia (field tests) and New York (chamber tests); in one of these tests (bimodal *vs* single-peaked call), and in another test not reported here (conspecific *vs* heterospecific pulse shape), the results were also the same as in tests of *H. versicolor* from Missouri and Texas (chamber tests in Missouri). Second, in the tests conducted in different setups there were large differences in the properties of alternative stimuli (two peaks *vs* one peak; high-frequency peak *vs* low-frequency peak) that were unlikely to have been obscured even under the less controlled field conditions. Third, the playback level (85 dB SPL) in all of the tests conducted in different setups and compared in this paper was well above the ambient background noise levels (<50 dB SPL, C-weighted). Tests in which the playback level of the alternative stimuli was lower than 85 dB SPL were all conducted in the chamber in Missouri. Finally, samples of females of *H. versicolor* from Missouri were tested in 2007 outdoors in a quiet park in the same field arena and equipment setup described above for tests of *H. avivoca*. Females showed the same preferences for the standard call as they did in the semi-anechoic chamber in the Missouri laboratory (bimodal call *vs* high-frequency peak alone [17-3]: G=0.94, d.f.=1, P=0.332; bimodal call *vs* low-frequency peak alone: [14-1] G=0.47, d.f. =1, P=0.49; high-frequency peak *vs* low-frequency peak [14-2]: G=0.63, d.f.=1, P=0.43).

Experimental procedure

Female gray treefrogs tested in the laboratory were collected in amplexus; many of these were refrigerated (about 4°C) to inhibit oviposition. Before testing (usually within 2 days of capture), each female was acclimated to the target test temperature (20°C) for at least 30 min. Field-tested females of *H. avivoca* and *H. versicolor* from Virginia were collected in amplexus, and after removal of the male, usually tested within several hours; a few females of *H. avivoca* were retained for testing on a subsequent night and were held in plastic boxes in a cooler with ice to retard oviposition. Females of *H. arenicolor* were collected along streams and in rock crevices in Arizona and transported by automobile to Missouri. They and a sample of females of *H. chrysoscelis* shipped by air from North Carolina to Missouri were later induced to respond phonotactically by the modified protocol of Schmidt (Schmidt, 1985) described elsewhere (Gerhardt, 1994). Neither refrigeration nor hormone induction of phonotaxis affects the selectivity of females of the gray treefrogs (Gerhardt, 1994) (N. M. Gordon and H.C.G., manuscript submitted)

Each female was placed in a circular, acoustically transparent (hardware cloth) cage located midway between the speakers. After each alternative stimulus had been played back several times in an alternating fashion, the top of the cage was removed. A response was tabulated when the female showed phonotactic orientation behavior (Rheinlaender et al., 1979) and moved to within 10 cm of one of the speakers. If a female did not begin phonotaxis within about 5 min or hopped away from the testing area within the chamber, the trial was recorded as a ‘no response’. Most responses occurred within 3 min of the female’s release. Each female provided a single datum for a test of a pair of alternatives. There was a minimum time-out of 2 min for females that were tested in more than one experiment. Control experiments have demonstrated that female treefrogs (*H. cinerea* and *H. versicolor*) were not biased for or against a stimulus they heard or responded to in a previous two-stimulus test (Gerhardt, 1981; Gerhardt et al., 2000). The loudspeakers playing back different alternatives were switched periodically within and between two-stimulus testing to minimize the effects of possible side biases; no such biases were detected (see also Gerhardt et al., 2000). Most females were released at the site of capture or held in the animal care facilities and used for other studies. These facilities and experimental procedures were approved by Animal Care and Use Committees of the University of Missouri, James Madison University and Pace University.

Our female-preference results are presented as the proportions of females that chose one of the alternatives (Figs 1–3). We computed the 95%-exact confidence limits using the F-distribution method employed in SAS Version 9.1. If a significant preference (two-tailed binomial, P<0.05) occurred, we show only one-sided limits unless otherwise noted; however, these P-values are not strictly valid because we had no explicit stopping rule. Moreover, the important biological information involves comparisons of proportions and overall patterns, rather than the statistical significance of any particular test. Accordingly, we report the appropriate statistical comparisons in the text and interpret the confidence limits as Bayesian credible intervals, which correspond numerically to confidence limits when the prior distribution is uniform [Gerhardt (Gerhardt, 1992) and references therein]. Note that credible intervals are interpreted differently than confidence limits. The true proportion is assumed have a 95% probability of being within the 95% credible limits, whereas if many confidence limits are computed, then 95% of them are expected to include the mean.

Results

We summarize the results of female preferences in tests of synthetic calls with two-peaked *versus* one-peaked spectra in Fig. 1. The data plotted for *H. versicolor* (NW mtDNA lineage)

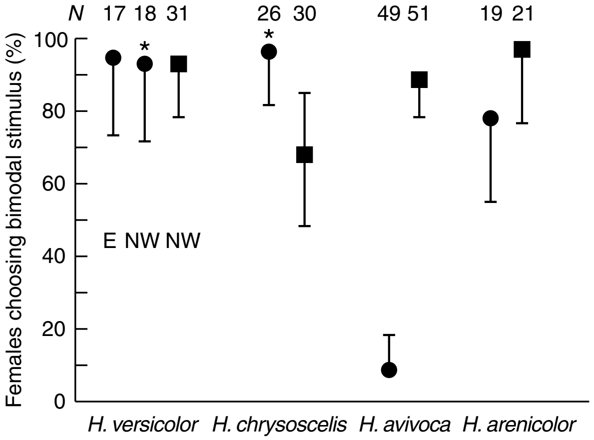

Fig. 1. Proportions of females choosing bimodal over unimodal alternatives. Numbers above symbols show the sample size (number of females responding). Circles, alternative to bimodal stimulus had only the high-frequency peak; squares, alternative to bimodal stimulus had only the low-frequency peak. SPLs equalized at 85 dB SPL except for tests of *H. avivoca*; the bimodal stimulus was 86 dB SPL and the single-peaked calls, 83 dB SPL (see the text for the rationale). Error bars are 95% credible intervals; if there is a single error bar, there was a significant ($P<0.05$) preference in a two-tailed binomial test. *Previously published data re-plotted (Gerhardt, 2005b); see text. NW, northwestern mtDNA lineage; E, eastern mtDNA lineage as defined by Ptacek et al. (Ptacek et al., 1994).

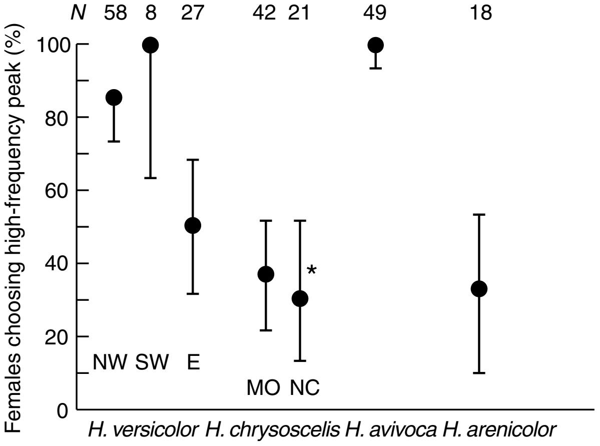

Fig. 2. Proportions of females choosing unimodal high-frequency calls over unimodal low-frequency calls at 85 dB SPL. NW, northwestern mtDNA lineage; SW, southwestern mtDNA lineage; E, eastern mtDNA lineage as defined by Ptacek et al. (Ptacek et al., 1994); NC, North Carolina; MO, Missouri. *The *P*-value of a two-tailed binomial test was 0.0495, even though the upper 95% credible limit was 52.2%, almost the same as that for the non-significant ($P>0.05$) result for tests of females from MO.

and *H. chrysoscelis* from Missouri were previously published [plotted as the percentage of females choosing the alternative with the high-frequency peak alone (Gerhardt, 2005a)] and are presented here for comparison.

We also show the results of new tests in which the bimodal stimulus was tested against an alternative having just the low-frequency peak. In *H. versicolor*, strong preferences for the bimodal stimulus occurred at 85 dB SPL; in *H. chrysoscelis*, there was strong but non-significant trend for a preference for the bimodal stimulus. Females of *H. arenicolor* also preferred calls with a bimodal spectrum to single-peaked alternatives. Females of *H. avivoca* discriminated against the stimulus with both peaks when the alternative had only the high-frequency peak. They chose the bimodal stimulus when the alternative had only the low-frequency peak.

Differences between mtDNA-defined lineages of *H. versicolor* occurred in the choices of females tested with single-peaked signals at 85 dB SPL (Fig. 2; $G=12.4$, d.f.=2, $P=0.002$). Two of the *H. versicolor* lineages (SW and NW) preferred the alternative with the high-frequency peak, whereas females of the E lineage (two populations) of *H. versicolor* did not show a preference. The lack of a preference was consistent in the two eastern populations: six of ten females from the VA population chose the low-frequency call in VA; nine of 20 from the NY population did so (not shown). Females of *H. avivoca* strongly preferred the alternative of high frequency, whereas the majority of females of *H. chrysoscelis* and *H. arenicolor* chose the alternative of low frequency. The latter preferences were either non-significant (*H. arenicolor* and *H. chrysoscelis* from Missouri) or marginally significant (*H. chrysoscelis* from North Carolina), and substantial minorities of the animals chose the high-frequency alternative (Fig. 2).

The preferences of females of *H. versicolor* and *H. chrysoscelis* from Missouri depended on the SPL at which alternatives of low and high frequency were equalized; data for tests at 85 dB SPL are repeated for comparative purposes (Fig. 3). In *H. versicolor* tested at 75 dB SPL, the original preference for the high-frequency alternative was reversed, and at 65 dB SPL, nearly all of the females chose the low-frequency alternative. The preference was also abolished when the SPL of the low-frequency call was held constant at 85 dB and that of the high-frequency call was reduced to 79 dB (not shown). In *H. chrysoscelis* tested at 75 dB SPL, there was a significant preference for the low-frequency peak, and at 65 dB SPL, the preference was unanimous. The differences in choices between playback levels of 85 and 65 dB SPL were statistically significant in both species (*H. versicolor*: $G=48.1$, d.f.=1, $P<0.0001$; *H. chrysoscelis*: $G=7.73$, d.f.=1, $P<0.01$).

Discussion

Matched filters and pre-existing biases

The pattern of preferences expressed by the four species is consistent with the matched-filter hypothesis: spectral patterns (bimodal or unimodal) typical of conspecific advertisement calls were more effective than other patterns. In *H. avivoca*, in which males produce calls with a single high-frequency peak, the addition of a low-frequency peak sharply reduced signal attractiveness relative to the single-peaked high-frequency alternative. Similar results were reported in the cricket frog (*Acris crepitans*) and the midwife toad (*Alytes cisternasii*), in which males also produce signals with a single spectral peak to which basilar papilla is tuned (Witte et al., 2001; Bosch and

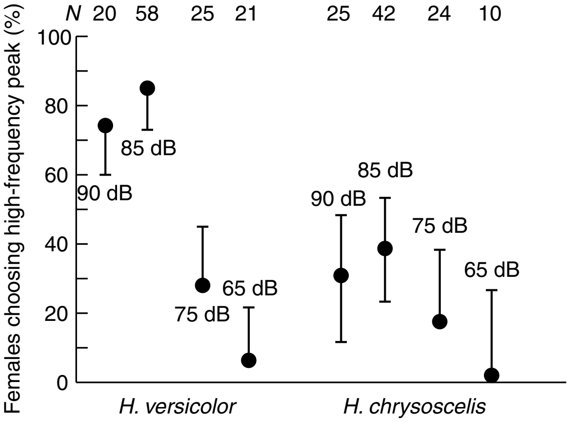

Fig. 3. Proportions of females of *H. versicolor* (NW mtDNA lineage) and *H. chrysoscelis* from Missouri choosing unimodal high-frequency calls over unimodal low-frequency calls at different playback levels.

Boyero, 2003). However, the overall SPL of the alternatives in those studies were equalized, so that the amplitude of the high-frequency component in the bimodal stimulus would have been about 3 dB less than the amplitude of the alternative signal having only the high-frequency peak. For this reason preferences for the single-peaked stimulus could also be interpreted as preferences for the stimulus that more effectively stimulated the basilar papilla.

In *H. avivoca*, the suppressive effect of low-frequency stimulation is unlikely to be strong because the bimodal stimulus was still attractive to females when the alternative was a single-peaked, low-frequency call. In other words, the extra stimulation did not inhibit phonotaxis. Nevertheless, the results of these playback experiments at biologically reasonable intensities as well as those of cricket frogs and midwife toads are opposite to the predictions of the pre-existing bias hypothesis in its broadest interpretation, namely that a novel signal providing extra sensory stimulation will be favored by females (Ryan and Rand, 1990). We emphasize that the results may differ at lower playback levels or if components were added of lower frequency than used in this study (<0.5 kHz, to which the amphibian papilla is also sensitive). Moreover, experiments with many other species producing calls with a single spectral peak are required before any broad generalization is warranted.

Broad-scale patterns of evolution

Another expectation of the matched-filter hypothesis is that geographical variation in the sensitivity of receivers will be concordant with geographical variation in signals. In cricket frogs (*Acris crepitans*), for example, the overall tuning as estimated from threshold measurements of auditory neurons innervating the basilar papilla or multi-unit activity in the torus semicircularis of males were similar to the dominant frequency of their calls (Wilczynski et al., 1992). Females in each population were tuned to and usually preferred signals with slightly lower-than-average frequencies, at the single playback level at which they were tested (Ryan et al., 1992).

Whereas a bimodal spectral structure is characteristic of the calls of all lineages of *H. versicolor* and the calls of *H. chrysoscelis* from throughout its the range of distribution, we found significant differences in preference in tests of low- *vs* high-frequency calls at a playback level of 85 dB SPL. Females of the NW and SW mtDNA lineages of *H. versicolor* preferred a single-peaked call of high frequency, whereas females of the E lineage did not show a preference. Females of *H. chrysoscelis* from eastern and Missouri populations also failed to show a preference, but preliminary experiments indicate that females of *H. chrysoscelis* from the extreme northwestern part of the range of distribution in Minnesota prefer the high-frequency call (M. A. Bee and H.C.G., unpublished data). Differences in preference among the three tetraploid lineages and between diploid taxa (*H. chrysoscelis, H. avivoca, H. arenicolor*) indicate that the ploidy level *per se* is unlikely to have affected the evolution of preferences based on spectral structure. These results suggest that it will be worthwhile to compare the different mtDNA lineages of *H. versicolor* to learn if there are also non-parallel changes between call structure and preferences based on fine-scale temporal properties as documented for *H. versicolor* and *H. chrysoscelis* from Missouri (Schul and Bush, 2002). That study and the work of Ryan and his colleagues (e.g. Ryan and Rand, 1990) attest to the fact that differences in preferences and underlying mechanisms can evolve independently of signals to some extent.

Neurobiological implications

The species difference in spectral-peak preferences between *H. chrysoscelis* and *H. versicolor* from localities in Missouri is abolished at the lowest playback level, and thus intensity-dependent changes in preference are likely to be general to this group of treefrogs. Indeed, because frequency and spectral-structure preferences have been found to be intensity-dependent in other anurans (e.g. *H. cinerea*) (Gerhardt, 1981; Gerhardt, 1987), we suggest that any study conducted at a single playback level is incomplete at best and may be misleading.

The effect of playback level on preferences was especially striking in females of NW *H. versicolor*, which strongly preferred the high-frequency call at 85 dB SPL and 90 dB SPL but reversed their preference at 75 dB SPL and 65 dB SPL. These results indicate a non-linear contribution of inputs from the two auditory organs or processing of those inputs in the ascending pathway as a function of increasing intensity. Regardless of the source(s) of the non-linearity, the influence on female choice of stimulation of the basilar papilla increases relative to that of amphibian papilla as intensity increases.

Neurophysiological studies of another treefrog species (*H. cinerea*) with a two-peaked advertisement call correlate well with behavioral results and are also consistent with the hypothesis of a non-linear convergence of input from the two auditory organs. Data from single auditory neurons at both the level of the eighth nerve (Capranica and Moffat, 1983) and torus semicircularis (Lombard and Straughan, 1974), and multi-unit audiograms from the torus semicircularis, show three regions of maximum sensitivity, two of which match the spectral peaks in the advertisement call. This species is much less sensitive in the high-frequency (basilar papilla) region than in the low-frequency (high end of the frequency range of the amphibian papilla) region. The difference in sensitivity correlates well with intensity-dependent changes in preferences for synthetic calls with bimodal spectra, preferences based on differences in the relative amplitudes of the two peaks, and preferences based on differences in frequency within these two regions of the spectrum (Gerhardt, 1981; Gerhardt, 1987). As in the gray treefrogs, the changes in behavioral preferences at high playback levels suggest an increasing influence of the input from the basilar papilla (high-frequencies in the call) as intensity increases to values of about 75 dB SPL and higher. These levels are probably typical of the call intensities that females usually experience as they approach males in choruses (Gerhardt, 1975); indeed, in one study of *H. cinerea*, females did not respond to the calls of a single male unless its SPL was the same (85 dB SPL) as a source of chorus background noise played at the same time and from the same direction (e.g. Gerhardt and Klump, 1988).

Two important differences exist between *H. cinerea* and the two species of gray treefrogs. First, the frequency difference between the two peaks is threefold in *H. cinerea* and only twofold in the gray treefrogs. This means that at high playback levels, energy primarily stimulating one of the two organs will be more likely to stimulate some of the receptors of the other organ in gray treefrogs than in *H. cinerea*. Cross-organ stimulation is likely to be at least part of the explanation for the intensity-dependent results in gray treefrogs (Hillery, 1984; Diekamp and Gerhardt, 1995; Gerhardt and Schul, 1999; Gerhardt, 2005b). Second, the two spectral peaks have about the same relative amplitude in *H. cinerea*, whereas the amplitude of the low-frequency spectral peak in gray treefrogs is normally about 10 dB less than that of the high-frequency peak (Gerhardt, 2005b). Nevertheless, previous research indicates that information carried by the low-frequency band has a strong positive influence on female selectivity for fine-scale temporal patterns such as pulse shape (Gerhardt and Schul, 1999). Moreover, bimodal stimuli in which the low-frequency peak was attenuated by as much as 30 dB relative to the high-frequency peak were still more attractive than an alternative stimulus with a single high-frequency peak (Gerhardt, 2005b). These results stand in stark contrast to the effect of additional low-frequency (AP) stimulation in *H. avivoca*, which had a negative effect on the relative attractiveness of a signal relative to an alternative with a single high-frequency peak.

Neurophysiological studies of the sensitivities of single auditory neurons innervating the two auditory organs of gray and bird-voiced treefrogs, as well as assessments of auditory responses at different stimulus playback levels in the ascending pathway, are necessary to identify the source(s) and loci of species and lineage differences in spectral preferences. Such studies may also be able to identify the mechanism(s) underlying the non-linear effects of increasing intensity on the relative influence of the two papillae and, along with behavioral experiments at different playback levels, are essential for understanding the proximate and ultimate factors that affect the evolution of acoustic communication at the population and species levels.

We thank R. Babb, S. Bisges, A. Bockhorst, T. Cook, D. Dittmer, A. Evers, N. Gordon, M. Hellman, C. Hoai, G. Höbel, K. Huth, N. Maximiv, C. McConkey, A. Miller, C. Plakosh, C. Tegtmeyer and M. Tucker for assistance in collecting and testing frogs. A. Feng and J. Schul provided comments on the manuscript. S. Humfeld provided general technical assistance, and N. Gordon administered the phonotaxis-inducing hormone protocol. Terri Slater of the US Forest Service helped to locate a study population in Virginia. This work was supported by the National Science Foundation (IBN-9873669 to C.G.M.; IBN-0091993 to H.C.G.), the Public Health Service (DHHS R01 DC05760 to H.C.G.) and the Department of Biology at James Madison University (to C.G.M.).

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