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Frequency channel-dependent selectivity for temporal call characteristics in gray treefrogs, *Hyla versicolor*

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ABSTRACT

Sensory receptors transmit information on multiple stimulus dimensions. Much remains to be understood about how the processing of different signal characteristics is partitioned and integrated in different areas of the nervous system. Amphibian hearing involves two morphologically distinct inner-ear organs that process different components of the frequency spectrum. Many anuran signals contain two frequency peaks, each one matching the sensitivity of one of these two organs. We hypothesized that the processing of temporal characteristics of acoustic signals would differ in these two frequency channels, perhaps because of differences in the response properties of the two inner-ear organs. We tested this hypothesis in the gray treefrog, *Hyla versicolor*; male advertisement calls of this species contain a bimodal frequency spectrum. We generated synthetic male advertisement calls in which we independently manipulated the pattern of amplitude modulation in the low-frequency peak or the high-frequency peak and measured the attractiveness of these stimuli to females in single-speaker and two-speaker phonotaxis tests. We obtained multiple lines of evidence that females were more selective for fine-temporal characteristics in the high-frequency peak. We discuss the potential implications of frequency channel-dependent temporal processing for signal evolution and suggest that additional neurophysiological investigations of the anuran auditory periphery will give important insights into how the nervous system partitions the encoding of multiple characteristics of complex signals.

KEY WORDS: Hearing, Anuran, Sensory processing, Amphibian papilla, Basilar papilla, Acoustic communication

INTRODUCTION

Even the simplest animal signals vary in multiple dimensions (Hebets et al., 2016). For instance, all acoustic signals, even those characterized as relatively invariant 'pure' tones (e.g. Beckers et al., 2003), necessarily have both a temporal and a spectral dimension, and most signals vary on multiple levels within these dimensions (Bradbury and Vehrencamp, 2011; Gerhardt and Huber, 2002). For any of this variation to be perceived and evaluated by receivers, it must first be encoded by sensory receptors (Bradbury and Vehrencamp, 2011). Information contained in the timing and rate of response of different sensory cells is passed on to higher processing centers in the nervous system, ultimately allowing receivers to evaluate relevant characteristics of signals for decision making (Gold and Shadlen, 2007). A challenge in all sensory processing is to identify signals as cohesive units amidst a background of noise in the form of any other stimulation that arrives simultaneously at the sensory receptors (Shinn-Cunningham, 2008). One means of accomplishing this is for nerve cells at different processing levels to act as filters, selectively responding only to stimuli with certain characteristics (von der Emde and Warrant, 2016; Wehner, 1987). However, this specialized filtering entails trade-offs: a given sensory cell may accurately transmit information about one stimulus dimension but may no longer represent well other stimulus dimensions (Bidelman and Syed Khaja, 2014; Fairhall et al., 2001; Henry et al., 2011). Much remains to be discovered about the partitioning of filtering characteristics among different components of sensory systems and how information from these filters is ultimately integrated.

The amphibian auditory system provides an exceptional opportunity to investigate how nervous systems evaluate multiple stimulus characteristics because of the unique architecture of the auditory receptors. Specifically, amphibians have two anatomically and physiologically distinct inner-ear organs specialized for detecting airborne sound, the amphibian papilla (AP) and the basilar papilla (BP) (Lewis and Narins, 1999; Simmons et al., 2007). These organs respond best to sounds of different frequencies: the AP to low-frequency sounds and the BP to higher frequency sounds. Individual fibers innervating the AP are narrowly tuned, although the organ as a whole can detect a broad frequency range because these fibers have different characteristic frequencies and are arranged tonotopically such that fibers with similar characteristic frequencies are located in spatial proximity to one another. In contrast, most individual BP fibers are somewhat more broadly tuned, but the organ as a whole generally detects a narrower frequency range because most fibers are tuned to a similar characteristic frequency (Smotherman and Narins, 2000). Correspondingly, the acoustic advertisement calls produced by many male anurans to attract mates and repel rivals often have bimodal frequency spectra such that one frequency peak falls within the range of responsiveness of the AP and the other closely matches the tuning of the BP (Gerhardt and Huber, 2002). Thus, frequency filtering already takes place in the auditory periphery. Temporal call characteristics are also important for species recognition, discrimination between different call types and determining the attractiveness of potential mates (Brenowitz and Rose, 1999; Gerhardt, 2008; Sullivan and Leek, 1987; Wells and Bard, 1987). Highly tuned temporal filters are well described in the auditory midbrain and higher processing centers in the brain (Rose and Gooler, 2007) but the possibility of temporal processing in the auditory periphery has received less attention (Schrode and Bee, 2015). Nonetheless, there is reason to expect differences between the two frequency channels of the auditory periphery in the processing of the pattern of amplitude modulation (AM) because of general trade-offs in auditory systems between frequency and time

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resolution (Gabor, 1946). Thus, fibers innervating the AP, which tend to be more narrowly tuned to specific frequencies, may have weaker resolution of AM rates than fibers innervating the BP, which tend to be more broadly tuned (Feng et al., 1991; Ronken, 1991; Simmons et al., 2007).

We investigated differences in the temporal processing of advertisement call characteristics between the frequency channels that best stimulate each of the two inner-ear organs in the eastern gray treefrog, *Hyla versicolor* LeConte 1825. *Hyla versicolor* was an ideal study subject for several reasons. First, male advertisement calls have a bimodal frequency spectrum (Fig. 1A), and each frequency peak is within the tuning range of one of the

two inner-ear organs (Diekamp and Gerhardt, 1995; Gerhardt, 2005). Second, temporal processing is critical for mating behavior in this species. Male advertisement calls consist of a series of pulses with species-specific shape, duration and repetition rate (Fig. 1C; Gerhardt, 2001). Female *H. versicolor* are especially sensitive to these characteristics and discriminate against calls with small deviations from species-typical values (Gerhardt and Doherty, 1988; Schul and Bush, 2002). Such discrimination is important in mate recognition because *H. versicolor* is sympatric with Cope's gray treefrog, *Hyla chrysoscelis* Cope 1880, throughout much of its range. These species' advertisement calls differ primarily in fine temporal structure (Fig. 1C–F;
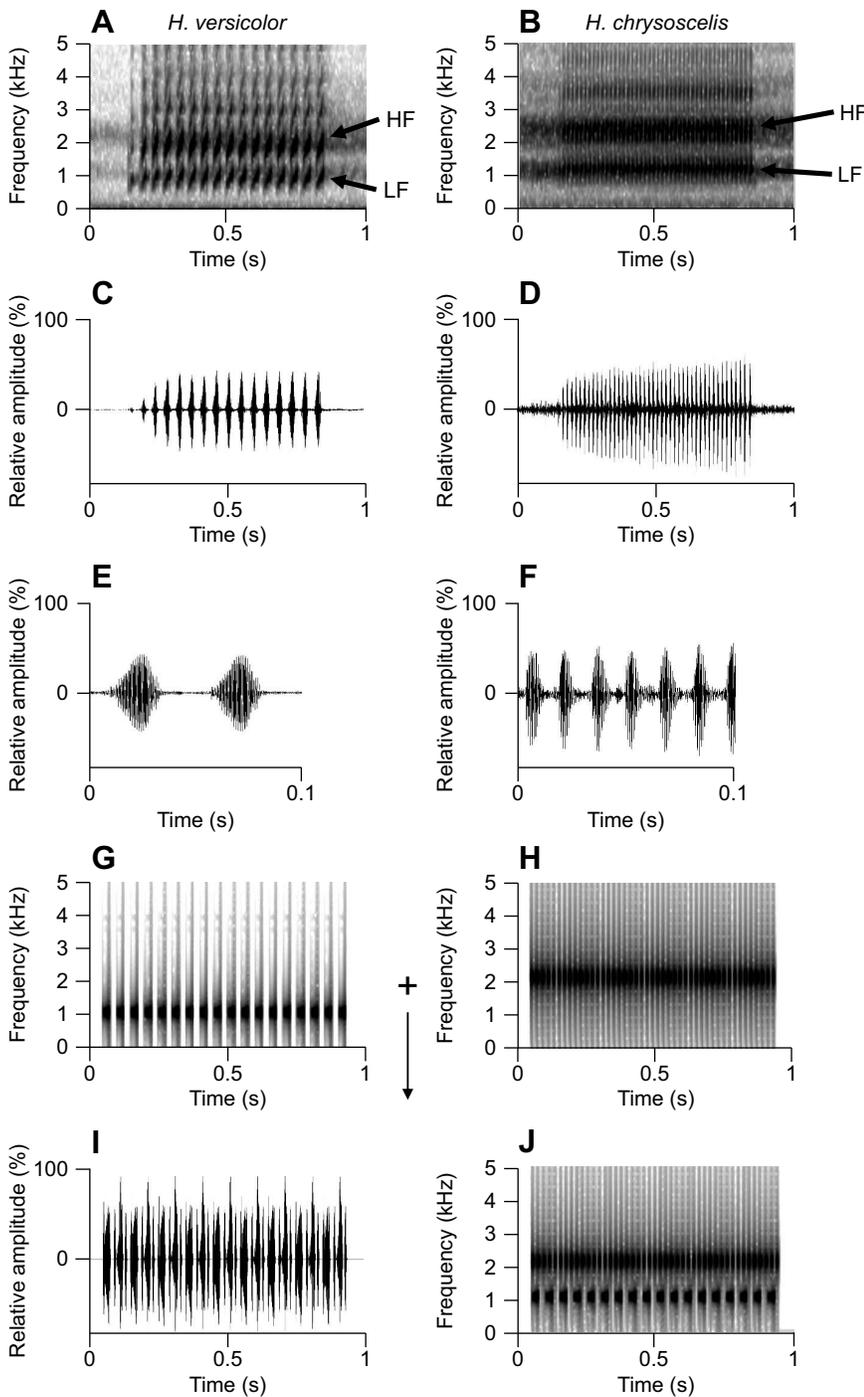

Fig. 1. Gray treefrog calls and synthetic stimuli. (A) A spectrogram of a recording of a natural male *Hyla versicolor* advertisement call illustrating the two frequency peaks (HF, high-frequency peak; LF, low-frequency peak). The amplitude of each frequency component at a given time is depicted by the darkness of the component on the grayscale. (B) A spectrogram of a recording of a natural male *Hyla chrysoscelis* advertisement call. *Hyla chrysoscelis* and *H. versicolor* calls have similar spectral structures but different patterns of amplitude modulation. (C) Waveform display of the same *H. versicolor* advertisement call depicted in A. (D) Waveform display of the same *H. chrysoscelis* advertisement call depicted in B. (E) A zoomed-in view of two individual pulses of the *H. versicolor* advertisement call from C. (F) A zoomed-in view of six individual pulses of the *H. chrysoscelis* advertisement call from D, illustrating the differences between the two species in pulse shape, pulse duration and pulse repetition rate. (G) A synthetic stimulus consisting of only the low-frequency component of a *H. versicolor* call. (H) A synthetic stimulus consisting of only the high-frequency component of a *H. chrysoscelis* call. To create synthetic stimuli with altered AM patterns, we added together stimuli representing each frequency peak, as in G and H, to generate a new stimulus. In the example shown here, the resulting stimulus (waveform in I, spectrogram in J) had a low-frequency peak with *H. versicolor* AM and a high-frequency peak with *H. chrysoscelis* AM.

Gerhardt and Doherty, 1988). Third, a previous study in which AM patterns were altered in artificial unimodal calls (i.e. calls with a single frequency peak) found that females discriminated more strongly in favor of conspecific AM at low stimulus frequencies than at high stimulus frequencies (Gerhardt, 2008). This suggests differences in temporal processing in the two frequency channels. Finally, temporal filtering is well understood at higher levels of the nervous system in *H. versicolor*. Recordings in the auditory midbrain revealed neurons that are highly selective for conspecific AM patterns (Diekamp and Gerhardt, 1995; Hanson et al., 2015; Rose et al., 1985, 2015). Less is known about the temporal selectivity of lower levels of the auditory system, but a study of auditory-evoked potentials in the closely related *H. chrysoscelis* demonstrated that the auditory periphery is also involved in temporal processing (Schrode and Bee, 2015).

We hypothesized that temporal selectivity is frequency channel dependent, possibly corresponding to differences in temporal processing in the peripheral auditory system. To test this hypothesis, we performed a series of playback tests in which we measured female responses to synthetic advertisement call stimuli resembling conspecific calls and stimuli in which the AM pattern was altered in one of the two frequency peaks of the conspecific call. We used multiple different alterations to the AM pattern to determine whether temporal selectivity was consistently greater in one frequency channel or the other, and whether some alterations of AM were more salient to receivers than others. In particular, we used stimuli that were expected to be unattractive because one of the frequency peaks was altered to resemble the calls of a heterospecific (Gerhardt and Doherty, 1988), or that contained gaps and therefore potentially interfered with the neural processes involved in temporal integration (Rose et al., 2015). A stimulus in which one frequency peak was replaced with a tone served as a measure of the importance of AM *per se* on temporal processing. We compared female responses to these altered calls in single-speaker tests at different sound pressure levels (SPLs), and used two-speaker playbacks to test female preferences for calls with altered AM in either the low- or high-frequency peak.

MATERIALS AND METHODS
Experiments were performed in May–June 2015 and 2016, during the natural breeding season of *H. versicolor*. Females were collected from ponds in Ozaukee County, WI, USA. No *H. chrysoscelis* have been detected at our study populations so we consider them to be allopatric, although records of *H. chrysoscelis* in neighboring counties suggest the possibility of sympatry in the recent past (Casper, 1996). We performed nightly searches for females in amplexus, which were brought to our laboratory and placed in containers on melting ice to prevent oviposition. Before testing, we acclimated females by placing them in a darkened box until they reached the test temperature of 20°C. After females were tested, they were marked with a subcutaneous fluorescent tag to prevent multiple testing of the same individuals. All experiments were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee (ACUC protocol 13-14 no. 38).

Stimulus preparation
We performed phonotaxis tests with synthetic acoustic stimuli in which we independently manipulated the AM pattern in each of the spectral peaks of the *H. versicolor* advertisement call. We used custom-written software provided by J. Schwartz (Schwartz, 1991) to generate a separate audio file for each spectral peak; these files

were then added together (Fig. 1G–J) and saved as 16-bit WAV files (20 kHz sampling rate) using the tuneR (http://r-forge.r-project.org/projects/tuner/) and seewave (Sueur et al., 2008) packages in R 3.1.2 software (http://www.R-project.org/). Our baseline stimulus was designed to replicate the average advertisement call characteristics of males in the study populations; the other stimuli differed from the baseline stimulus in the AM pattern in one or both of the spectral peaks. For all stimuli, the low-frequency peak was at 1.07 kHz and the high-frequency peak was at 2.14 kHz. The two frequency peaks were broadcast at equal amplitude. Although the high-frequency peak is typically louder than the low-frequency peak in *H. versicolor* (Gerhardt, 1982, 2005), for comparing the temporal selectivity of the two frequency channels, it was important that we present stimuli with equal peak SPLs in the two frequency peaks. Preliminary analyses showed no difference in female responsiveness to calls with a 0 or 6 dB amplitude difference between the high- and low-frequency peak (M.S.R. and G.H., unpublished data). All stimuli had a total duration of 900 ms and were repeated with a call period of 7.55 s. For two-speaker tests, we manipulated the stimuli in Audacity 2.02 software (http://audacity.sourceforge.net/) so that each stimulus was broadcast from a single speaker channel and the stimuli were timed to be in perfect alternation with one another.

We generated the following types of single-frequency call components that differed in AM and that were combined to create the experimental stimuli (full list of stimuli in Table S1). (1) Typical *H. versicolor* (Fig. 1G). This component had the pulse rate and pulse shape characteristic of males in our study population (number of pulses 18, pulse duration 25 ms, inter-pulse interval 25 ms, pulse rise time 20 ms, pulse fall time 5 ms, linear pulse rise and fall). We refer to frequency components with this pattern as having conspecific AM. A call with conspecific AM in both frequency peaks is referred to as the 'conspecific call'. (2) Typical *H. chrysoscelis* (Fig. 1H). This component had the pulse rate and pulse shape characteristic of male *H. chrysoscelis* (number of pulses 45, pulse duration 10 ms, inter-pulse interval 10 ms, pulse rise time 5 ms, pulse fall time 5 ms, negative exponential pulse rise and fall). We refer to frequency components with this pattern as having heterospecific AM. (3) *Hyla chrysoscelis* pulse shape. This component had the pulse rate of *H. versicolor* but the pulse duration and shape of *H. chrysoscelis*. The inter-pulse interval was 40 ms, to maintain the *H. versicolor*-like pulse rate of 20 pulses s^{-1}. Females are expected to avoid heterospecific calls to prevent mismatings (Gerhardt and Doherty, 1988). The component with *H. chrysoscelis* pulse shape and duration was used because *H. versicolor* primarily evaluate these characteristics rather than pulse rate (Gerhardt and Schul, 1999; Schul and Bush, 2002); we tested whether such fine discriminations are possible when presented in only one frequency channel. (4) Gappy *H. versicolor*. This component was identical to the 'typical *H. versicolor*' stimulus except that for every 3 pulses broadcast, 3 pulses were silenced. Temporal filters in the anuran auditory system are especially sensitive to gaps within calls, which reset the process of temporal integration (Henderson and Gerhardt, 2013; Rose et al., 2015; Schwartz et al., 2010). (5) Tone. This component had the same total duration and frequency as the other stimuli, but was a pure tone with no AM other than a 5 ms linear rise and fall time.

Test procedure
All experiments were performed in a darkened semi-anechoic chamber (for further details of the testing arena, see Reichert et al., 2014). Females were placed in an acoustically transparent release

cage in a circular arena 1 m from each of the playback speakers. We broadcast test stimuli through one or both of two speakers (JBL Control 1Xtreme, Harman International Industries, Inc., Stamford, CT, USA) oriented 90 deg apart from one another along the arena's edge, facing towards its center. We calibrated the SPL of stimulus broadcast by adjusting the speakers so that the conspecific call was broadcast at 85 dB SPL [fast root mean square (RMS), C-weighting, re. 20 μPa] measured with an Extech 407764 SPL meter (Extech Instruments Corporation, Nashua, NH, USA). We then measured the peak SPL of the same stimulus and, except where noted below, broadcast all other stimuli at this peak amplitude. Stimuli therefore did not necessarily have the same RMS amplitude because of differences in duty cycle arising from differences in AM. We chose this approach to equalize the absolute stimulus amplitude presented in each frequency channel. This was readily achieved by equalizing peak SPL, while adjustments of RMS amplitude for stimuli containing multiple AM patterns may have differentially affected the relative representation of frequency channels in the playback. In any event, the most important comparisons for this study were between stimuli with identical sound energies, differing only in the frequency channel in which the altered AM pattern was presented.

We broadcast one repetition of each call of the selected test stimulus before remotely lifting the release cage, allowing the female to move freely about the arena. We monitored and recorded female movements using an overhead infrared video camera. A choice was defined as an approach to within 10 cm of one of the speakers. Females that did not make a choice within 5 min or that wandered through the choice zone incidentally while moving along the arena's walls were considered non-responders in the trial. For single-speaker tests, we also measured the female's latency to approach as the amount of time between her release into the arena and her first entry into the choice zone. Females respond more rapidly to more attractive stimuli (Bush et al., 2002; Reichert and Höbel, 2015). Females were tested with multiple stimuli. Stimulus presentation order was randomized and we switched speaker locations periodically to control for side biases. We tested a total of 92 females. Each individual female was tested with multiple experimental stimuli (mean trials per female 12.8, range 3–16). Females were given no time-out period between trials to ensure that we tested highly motivated females; there is no evidence for carry-over effects of multiple testing in *H. versicolor* (Gerhardt and Doherty, 1988).

Experiment 1: frequency channel-dependent temporal processing

Our primary aim was to test the hypothesis that temporal signal characteristics are processed differently in the two main frequency channels of the receiver's auditory system. We therefore gave females the choice between a stimulus with an altered low-frequency peak and one with a high-frequency peak altered in the same way. These calls were thus identical in temporal characteristics, but the AM patterns were swapped between the frequency channels of the alternatives, allowing us to determine whether selectivity for temporal characteristics was stronger in one frequency channel or the other. We tested the effects of several different alterations to the AM pattern to determine whether frequency channel-dependent temporal processing is a general phenomenon or instead is restricted to only specific AM patterns (see stimulus waveform schematic representations in Fig. 2; Table S1). An important assumption of our tests of the hypothesis of frequency-channel dependent temporal selectivity is that females do not have an inherent preference for one frequency peak over the other, perhaps because one peak is perceived as louder or better
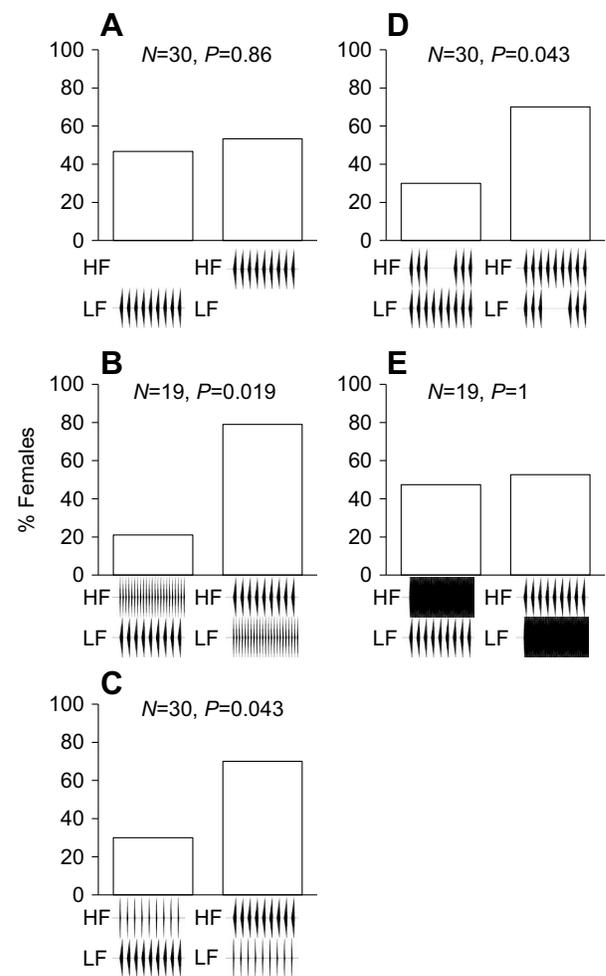

Fig. 2. Evidence for frequency-dependent temporal processing from experiment 1. Bars indicate the percentage of females that chose each stimulus. A schematic illustration of the stimulus alternatives in each two-speaker trial is given at the bottom of each panel. Note that for ease of interpretation, we illustrate the waveform of the AM pattern separately for the low-frequency peak (LF) and the high-frequency peak (HF); the actual stimulus waveform combined these two peaks to generate a single stimulus (see Fig. 1I). In addition, we only depict a 450 ms portion of each call in order to illustrate clearly the differences between the stimuli in AM. (A) The proportion of females choosing a call with a single frequency peak with conspecific AM in either the low- (left) or high-frequency peak (right). In the remaining graphs, the alternative depicted on the left contained *H. versicolor* AM in the low-frequency peak and AM in the high-frequency peak that was altered to have one of the following characteristics: (B) *H. chrysoscelis* AM, (C) *H. chrysoscelis* pulse shape and pulse duration, (D) gaps (missing pulses), (E) tone. The alternative depicted on the right had the opposite pattern: the same alteration of AM in the low-frequency peak and *H. versicolor* AM in the high-frequency peak. The total number of females tested and the *P*-value from a two-tailed binomial test of the null hypothesis of equal choices of both alternatives are shown above each graph.

stimulates both inner ear organs. Therefore, we gave females single-speaker tests with a stimulus containing only either the low- or the high-frequency peak of the conspecific call, along with a two-speaker test giving females the choice between these two stimuli.

Experiment 2: effects of heterospecific components on call attractiveness

Discriminating between conspecific and heterospecific calls is critical for successful mating; thus, alterations of the AM pattern

resembling heterospecific AM should be especially salient to choosing females. We used single-speaker tests to investigate how readily females responded to a call with conspecific AM in the low-frequency peak and heterospecific AM in the high-frequency peak, and a call with heterospecific AM in the low-frequency peak and conspecific AM in the high-frequency peak, relative to their response to the conspecific call. We performed these tests at three different SPLs: 85, 65 and 56 dB. The lower SPLs were used to reduce crosstalk between the auditory receptors at high stimulus intensities. That is, although auditory receptors have a best excitatory frequency with a low response threshold, they also respond to higher and lower frequencies if stimuli at these frequencies are loud enough (Gerhardt and Schwartz, 2001). The typical amplitude threshold for response is much lower than our standard experimental SPL of 85 dB (Diekamp and Gerhardt, 1995). Thus, it is probable that loud stimuli with conspecific AM in only one of the two frequency channels would nonetheless stimulate auditory receptors in both inner-ear organs. This effect could prevent us from detecting differential temporal selectivity based on frequency channel. We therefore also measured female responses to these stimuli at two lower SPLs, selected based on preliminary tests of female responsiveness to calls at these amplitudes. Nevertheless, even these stimuli may have been above the threshold amplitude of some auditory neurons, and amplitude is further increased as females move towards the speaker; thus, we cannot rule out some effect of crosstalk on our results.

We also performed a series of two-speaker playback tests, giving females the choice between the conspecific call and calls with heterospecific AM (or heterospecific pulse shape, but conspecific pulse rate) in either the low- or high-frequency peak, and conspecific AM in the other peak. Together, these four tests allowed us to determine whether heterospecific AM, which is normally unattractive to females, is also unattractive when present in only one of the two frequency peaks. We also tested whether calls with a bimodal frequency spectrum, even if one peak has heterospecific AM, are more attractive than calls with a single frequency peak (Gerhardt, 2005). We therefore performed four tests in which females were given the choice between either the low- or the high-frequency peak of the conspecific call (other peak missing) and a call with heterospecific AM in either the low- or the high-frequency peak, and conspecific AM in the other peak (Table S1).

Experiment 3: effects of tones on call attractiveness
The tone stimulus has no AM and was used to determine whether there is a difference in female response to stimuli with individual frequency components either with or without AM. We used single-speaker tests to investigate the attractiveness of a call with conspecific AM in the low-frequency peak and a tone in the high-frequency peak, and a call with a tone in the low-frequency peak and conspecific AM in the high-frequency peak, relative to the conspecific call. As in experiment 2, we performed these trials at three different SPLs: 85, 65 and 56 dB. We also performed two-speaker tests, giving females the choice between the conspecific call and calls with a tone in either the low- or high-frequency peak, and conspecific AM in the other peak.

Experiment 4: effects of gaps on call attractiveness
Female *H. versicolor* discriminate against calls with missing pulses (Schwartz et al., 2010). In this experiment, we asked whether females would also discriminate against calls in which pulses were missing in only one of the two frequency channels, and whether this discrimination is frequency channel dependent. We tested females

in a series of two-speaker playback tests in which at least one of the stimuli contained gaps (i.e. for every 3 pulses broadcast, the 3 subsequent pulses were missing, otherwise the AM was as the conspecific call) in at least one of the frequency peaks (Table S1).

Statistical analyses
For each of the two-speaker tests, we tabulated the number of females that chose each stimulus and used a two-tailed binomial test with a null expectation of 50% choices to each stimulus to determine whether females significantly preferred one stimulus over the other. For the single-speaker tests of experiments 2 and 3, we compared the number of females that responded or failed to respond to the conspecific call and to the stimuli with altered AM using generalized linear mixed models with response or non-response as the binary dependent variable (logit link function), the stimulus, SPL and the interaction between stimulus and SPL as fixed effects, and female identity as a random effect. We coded SPL as a categorical variable. For the stimulus variable, the conspecific call was the reference factor level, allowing us to determine whether altering the AM in one of the frequency channels affected female responses relative to their response to the conspecific call. Separate tests were performed for experiments 2 and 3. We also compared females' response latencies for the single-speaker experiments using linear mixed models with stimulus, SPL and the interaction between stimulus and SPL as fixed factors, and female identity as a random factor. Only trials in which the female responded to the stimulus were included in the analyses of response latency. Again, separate tests were performed for experiments 2 and 3. When the interaction term was not statistically significant ($P>0.05$), it was removed from the model. Mixed models were performed in the lme4 package (Bates et al., 2015) in R 3.2.2 software. All statistical tests were two-tailed with alpha=0.05.

RESULTS
Experiment 1: frequency channel-dependent temporal processing
Overall, results of this experiment support the hypothesis of frequency channel-dependent temporal selectivity, and point towards a dominance of the high-frequency peak. In single-speaker tests, females nearly always responded to stimuli containing only the low- or the high-frequency peak of the conspecific call (19/20 females responded to the low-frequency peak; 20/20 females responded to the high-frequency peak). In a two-speaker test, females had no preference for the low-frequency peak or the high-frequency peak of the conspecific call (Fig. 2A). When given a choice between calls with conspecific AM in one frequency peak and altered AM in the other frequency peak, females usually preferred calls with the conspecific AM in the high-frequency peak and altered AM in the low-frequency peak to calls with the opposite arrangement, supporting the hypothesis of frequency channel-dependent temporal selectivity. This was true for calls in which the AM-altered frequency peak contained heterospecific AM (Fig. 2B), *H. versicolor* pulse rate but *H. chrysoscelis* pulse shape (Fig. 2C), and gaps (missing pulses; Fig. 2D). However, females showed no preference when the altered frequency peak contained a tone (Fig. 2E).

Experiment 2: effects of heterospecific components on call attractiveness
Females were surprisingly responsive to calls in which AM was altered in one of the two frequency channels to resemble that of *H. chrysoscelis*, but whenever a negative effect of heterospecific

components was evident, it involved manipulation of the high-frequency peak. For single-speaker playbacks at 85 dB, nearly all females responded to both the conspecific call and stimuli with heterospecific AM in either peak (Fig. 3A). At lower playback SPLs, however, females were somewhat less likely to respond, and response likelihood was conspicuously lower for the stimulus with heterospecific AM in the high-frequency peak (Table 1, Fig. 3A). Females responded most quickly to stimuli presented at 85 dB, but there were no differences in response latencies to the conspecific call and to calls with heterospecific AM in one of the two frequency peaks (Table 2, Fig. 3B). However, fewer females responded at all to altered AM stimuli, thus providing relatively few samples and correspondingly reduced statistical power to detect effects on response latencies.

Females significantly preferred the conspecific call to a call with heterospecific AM in the high-frequency peak (Fig. 4A), but they did not prefer the conspecific call to a call with heterospecific AM in
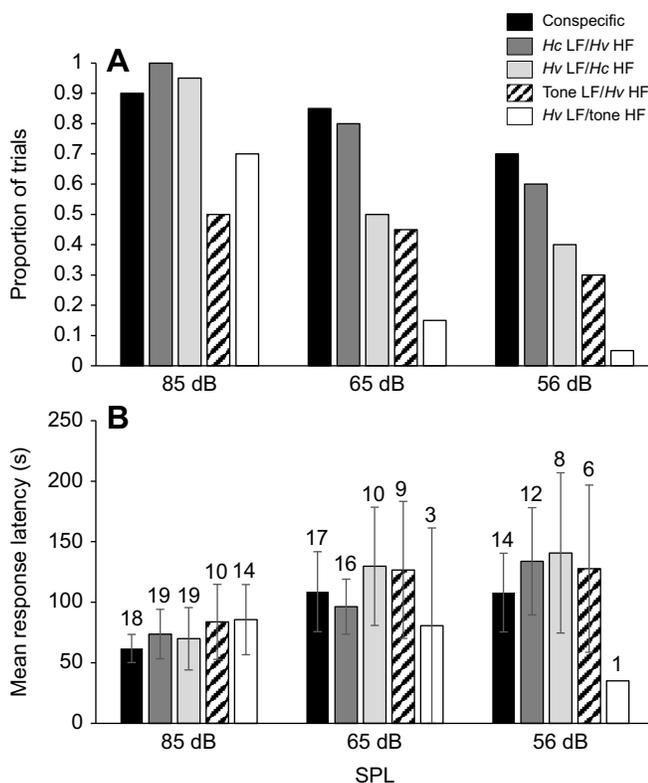

Fig. 3. Female response in single-stimulus trials. (A) Female response is illustrated as a proportion of trials responded to, calculated as the number of females that approached the speaker within 300 s out of the total number of females tested with that stimulus (N=20 females tested per stimulus, except for Hc LF/Hv HF at 85 dB, where N=19). (B) Mean±95% confidence interval of female latency to approach the speaker. Numbers above bars indicate the number of females that responded to the stimuli; we did not include latency values for females that did not respond. There is no error bar for the Hv LF/tone HF stimulus at 56 dB because only one female responded to this stimulus. Female responses were measured for five different stimuli (conspecific, the normal synthetic H. versicolor advertisement call; Hc LF/Hv HF, a call with H. chrysoscelis AM in the low-frequency peak and H. versicolor AM in the high-frequency peak; Hv LF/Hc HF, a call with H. versicolor AM in the low-frequency peak and H. chrysoscelis AM in the high-frequency peak; tone LF/Hv HF, a call with a tone in the low-frequency peak and H. versicolor AM in the high-frequency peak; Hv LF/tone HF, a call with H. versicolor AM in the low-frequency peak and a tone in the high-frequency peak) at three different sound pressure levels (SPLs).

Table 1. Results of generalized linear mixed models of female response in single-speaker trials

Effect	Estimate	s.e.m.	Z	P
Experiment 2				
Intercept	3.87	0.81	4.8	**<0.001**
Stimulus (conspecific AM, LF/heterospecific AM, HF)	−1.34	0.50	−2.7	**0.008**
Stimulus (heterospecific AM, LF/conspecific AM, HF)	−0.14	0.52	−0.3	0.79
SPL (56 dB)	−3.06	0.78	−3.9	**<0.001**
SPL (65 dB)	−2.27	0.76	−3.0	**0.003**
Experiment 3				
Intercept	2.95	0.58	5.1	**<0.001**
Stimulus (conspecific AM, LF/tone, HF)	−2.90	0.55	−5.3	**<0.001**
Stimulus (tone, LF/conspecific AM, HF)	−2.26	0.51	−4.4	**<0.001**
SPL (56 dB)	−2.06	0.55	−3.7	**<0.001**
SPL (65 dB)	−1.26	0.52	−2.4	**0.015**

The effect estimate, s.e.m. and statistical tests of the effect (Z, P) of stimulus and sound pressure level (SPL) on the number of females that responded in the single-speaker trials of experiment 2, in which one of the two frequency channels contained heterospecific amplitude modulation (AM), and experiment 3, in which one of the two frequency channels contained a tone. The reference value for stimulus is the conspecific call; the reference value for SPL is 85 dB. HF, high frequency; LF, low frequency.

the low-frequency peak (Fig. 4B). Likewise, females significantly preferred the conspecific call to a call with H. versicolor pulse rate but H. chrysoscelis pulse duration and shape when the modification was to the high-frequency peak (Fig. 4C) but not when it was to the low-frequency peak (Fig. 4D). Adding heterospecific AM to a unimodal conspecific frequency component neither increased nor decreased the call's attractiveness: when given the choice between a call with a single conspecific frequency peak and a call with conspecific AM on one peak and heterospecific AM on the other, females showed no significant preference for either stimulus (Fig. 5).

Table 2. Results of linear mixed models of female response latency in single-speaker trials

Effect	Estimate	s.e.m.	d.f.	t	P
Experiment 2					
Intercept	117.92	10.76	117	11.0	**<0.001**
Stimulus (conspecific AM, LF/heterospecific AM, HF)	17.14	11.57	108	1.5	0.14
Stimulus (heterospecific AM, LF/conspecific AM, HF)	7.37	10.78	107	0.7	0.5
SPL (56 dB)	−15.29	12.14	121	−1.3	0.21
SPL (65 dB)	−57.66	11.74	73	−4.9	**<0.001**
Experiment 3					
Intercept	111.83	12.26	76	9.1	**<0.001**
Stimulus (conspecific AM, LF/tone, HF)	10.00	12.33	65	0.8	0.42
Stimulus (tone, LF/conspecific AM, HF)	23.61	10.51	66	2.2	**0.03**
SPL (56 dB)	−6.93	12.57	72	−0.6	0.58
SPL (65 dB)	−45.71	15.75	63	−2.9	**0.005**

The effect estimate, s.e.m. and statistical tests of the effect (t, P) of stimulus and SPL on female response latency (larger values=slower response) in the single-speaker trials of experiment 2, in which one of the two frequency channels contained heterospecific AM, and experiment 3, in which one of the two frequency channels contained a tone. The reference value for stimulus is the conspecific call; the reference value for SPL is 85 dB.
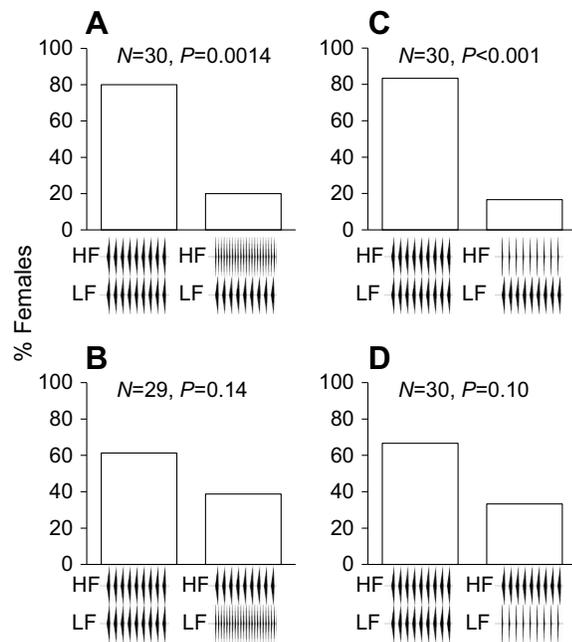
Fig. 4. Female response in two-speaker trials in experiment 2. Interpretations of graphs and call illustrations as in Fig. 2. One call (depicted on the left) was the conspecific call in all cases. The other call (depicted on the right) was altered to have *H. chrysoscelis* AM in either the high-frequency peak (A) or low-frequency peak (B) or pulses with the shape and duration of *H. chrysoscelis*, but the repetition rate of *H. versicolor* in either the high-frequency peak (C) or low-frequency peak (D).

Fig. 5. Female response in two-speaker trials with unimodal or bimodal frequency spectra in experiment 2. Interpretations of graphs and call illustrations as in Fig. 2. In these trials, one call (depicted on the left) was unimodal in that it contained only one of the two frequency peaks; this peak had *H. versicolor* AM. The other call (depicted on the right) had a bimodal frequency spectrum, in which one frequency peak had *H. versicolor* AM and the other frequency peak had *H. chrysoscelis* AM.

Experiment 3: effects of tones on call attractiveness

Calls in which conspecific AM was replaced with a tone were generally unattractive. In single-speaker trials at 85 dB, females responded less often to calls with a tone in one of the frequency peaks than they did to the conspecific call (Table 1, Fig. 3A). At lower playback SPLs, females responded even less to such stimuli, and tones were unattractive regardless of whether they were inserted into the high- or low-frequency peak (Table 1, Fig. 3A). Females responded more slowly to tone stimuli at lower playback SPLs (Table 2, Fig. 3B), but very few females responded to these stimuli at all, so comparisons of response latencies are limited by low statistical power. In two-speaker tests, females had a strong preference for the conspecific call over calls with a tone in either of the two frequency peaks and conspecific AM in the other peak (Fig. 6).

Experiment 4: effects of gaps on call attractiveness

Gappy calls were unattractive but females still responded frequently when gaps were confined to only one of the two frequency peaks. Females unanimously chose the conspecific call over a call with missing pulses in both frequency channels (Fig. 7A). Females also preferred a conspecific call over a call in which the gaps were confined to one of the two frequency peaks, although this relationship was only significant for the call with gaps in the high-frequency peak (Fig. 7B,C). Females strongly preferred calls with gaps in only one of the two frequency peaks to calls with gaps in both frequency peaks (Fig. 7D,E).

DISCUSSION

We obtained multiple lines of evidence that females are more selective to variation in fine-temporal characteristics of advertisement calls in the high-frequency channel, processed by
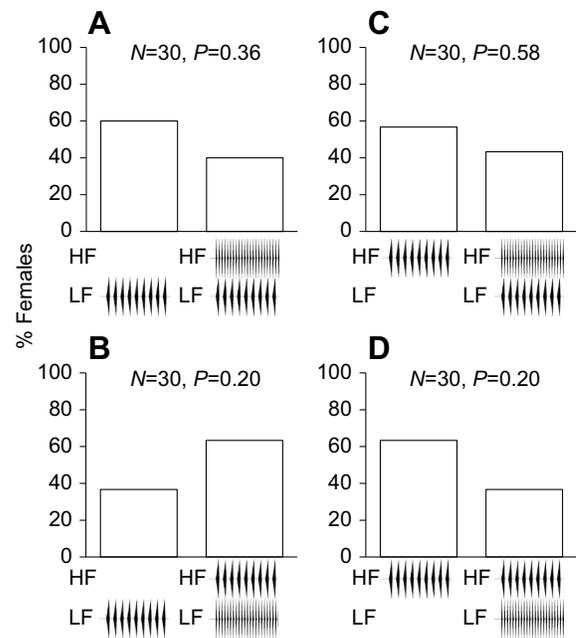
the BP, than the low-frequency channel, processed by the AP. In single-stimulus tests at low SPL, females responded more often to calls with heterospecific AM or tones in the low-frequency peak and conspecific AM in the high-frequency peak than they did to calls with the opposite arrangement. In two-speaker tests in which alternatives had altered AM in either the low- or high-frequency peak, females preferred calls with a conspecific high-frequency peak and altered AM in the low-frequency peak over calls with the opposite arrangement. The exception to this pattern was for calls with a tone in one frequency peak, in which females showed no preference, and to which females were generally not attracted. Our results are not likely to be attributable to inherent biases in response towards one frequency component over the other that could have arisen because one component is more effective at stimulating one
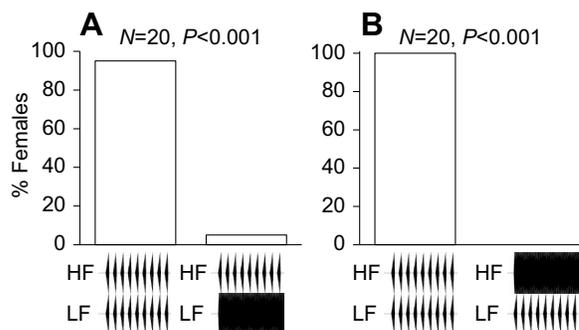

Fig. 6. Female response in two-speaker trials in experiment 3. Interpretations of graphs and call illustrations as in Fig. 2. In these trials, one call (depicted on the left) was the conspecific stimulus. The other call (depicted on the right) contained a tone in either (A) the low-frequency peak or (B) the high-frequency peak, in place of conspecific AM in that peak.
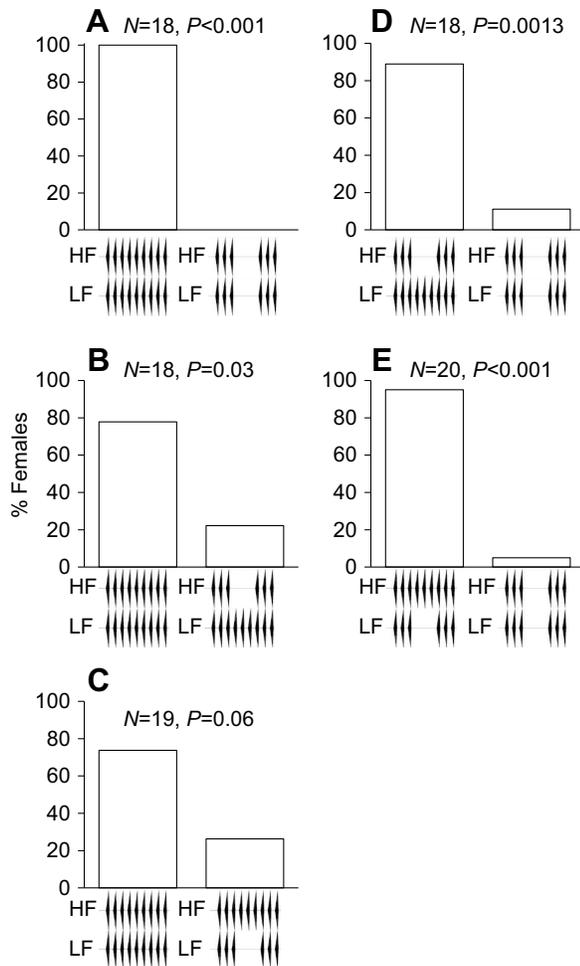
Fig. 7. Female preference for gappy calls in experiment 4. Interpretations of graphs and call illustrations as in Fig. 2. In these trials, the alternatives varied in whether either or both of the frequency peaks were altered to contain gaps (missing pulses). Remaining frequency peaks contained *H. versicolor* AM.

or both of the inner ear organs: in a two-speaker test, females showed no preference for the low- over the high-frequency peak of the conspecific call. Although we obtained multiple lines of evidence indicating the importance of the high-frequency channel in AM processing, with fine discrimination among call elements that differed in pulse rate or shape, female preferences were far from unanimous. Thus, females are rather permissive in their responses provided that at least one frequency peak has the conspecific AM pattern. Nevertheless, the finding of differences in temporal processing between frequency channels has important consequences for both the understanding of the neural processing of acoustic signals and the evolution of complex signals.

Temporal and frequency filters in the nervous system
The understanding of temporal processing in early stages of the anuran auditory system is still relatively limited compared with knowledge of frequency selectivity at these stages (Lewis and Narins, 1999; Simmons et al., 2007). Nonetheless, previous studies (e.g. Schrode and Bee, 2015) and our own results indicate that some temporal processing takes place before information from the two frequency channels is integrated in the central nervous system. Our finding of differences in behavioral selectivity for temporal characteristics based on frequency channel raises important

questions about how different components of the auditory system partition the processing of complex signal characteristics, and how this information is ultimately integrated. It is clear that both frequency channels process AM, but if one is indeed more selective for AM pattern, is its output weighted more heavily when temporal information from the two channels is integrated? Is variation in temporal selectivity across frequency channels solely due to trade-offs between spectral and temporal resolution (Henry et al., 2011) arising from the anatomy and physiology of the two inner-ear organs and their associated neurons, or is it also a result of selection on communication in particular ecological and social conditions (see below)? Further physiological investigations of temporal filtering in sensory receptors and neurons in the auditory periphery, and of the relationships between these processes and frequency filtering are clearly needed. In general, knowledge of the mechanisms of sensory processing of multidimensional signal characteristics is relatively limited compared with what is known about the behavioral consequences of complex signaling in non-human animals (Partan, 2013).

The results of experiment 4 give new insights into the importance of interval-counting neurons on call recognition. These neurons, described in multiple anuran species, are only responsive to signals with a minimum number of correctly spaced sound pulses; signals containing gaps reset the process of temporal integration (Edwards et al., 2002; Rose et al., 2015). Correspondingly, we found that stimuli containing gaps were highly unattractive to females. However, the attractiveness of gappy stimuli was partly restored if the gaps were confined to only one of the two frequency channels. Furthermore, females preferred calls with gaps in the low-frequency peak to calls with gaps in the high-frequency peak. This suggests that interval-counting neurons may be more prevalent, or more sensitive, in the high-frequency channel. However, most interval-counting neurons have best excitatory frequencies well below those of either frequency peak in *H. versicolor* calls (Rose et al., 2015), so the relationship between frequency processing and temporal integration remains unclear and requires further investigation.

Our results differ strikingly from a previous study of *H. versicolor*, in which females were found to be more selective for differences in pulse rate at low rather than high frequencies (Gerhardt, 2008; see also Gerhardt and Schul, 1999). One explanation for this discrepancy is that most of the stimuli used by Gerhardt (2008) contained spectral energy in only one of the two frequency peaks. In contrast, most of our stimuli contained energy in both peaks, and conspecific AM in at least one peak. Most of our stimuli were presented at 85 dB, well above the threshold of auditory receptors in both frequency channels. Thus, an unattractive AM pattern in one frequency channel may have masked an attractive AM pattern in the other channel if both patterns were perceived in the same inner-ear organ because of crosstalk stemming from above-threshold stimulation. However, we consider this to be an unlikely general explanation for our results because females discriminated against calls with gaps in one channel and conspecific AM in the other (Fig. 7B,C); in these stimuli, pulses in the two frequency channels were synchronous, eliminating the possibility of masking of pulse pattern. Additional evidence that masking was not a major factor includes our finding that females did not discriminate between unimodal conspecific calls and bimodal calls containing heterospecific AM (Fig. 5), and that differences in responses to stimuli with altered AM in either the low- or high-frequency peak became more pronounced at lower stimulus amplitudes (Fig. 3A). Physiological studies of the response of neurons with different characteristic frequencies to our experimental

stimuli would be valuable to further explore the possible influence of masking. Another explanation for the discrepancy between studies is that Gerhardt (2008) found that frequency channel-dependent temporal selectivity depended on experimental conditions: in most tests, females were more selective to low-frequency stimuli, but at high SPLs, females were sometimes more selective to high-frequency stimuli. In contrast, our results consistently pointed towards the high-frequency channel as being most important for temporal selectivity, including in single-speaker tests at low playback SPLs. Nonetheless, we performed most tests at a single SPL, so we cannot rule out the possibility that we would have obtained different results at different SPLs.

Implications for signal evolution

The processing of both temporal and spectral information is necessary for effective acoustic communication, and receiver assessments of variation in both types of characteristics are important in anuran social interactions (Bee et al., 2016; Gerhardt and Bee, 2007; Gerhardt and Huber, 2002). We found that temporal selectivity is stronger at high frequencies than at low frequencies, which implies that selection mediated by female mate choice on fine-temporal call structure is weaker on the low-frequency peak. Thus, evaluations of temporal and spectral characteristics are not entirely independent processes. Is there any functional relevance to this frequency channel-dependent temporal selectivity for the sexual communication system? The frequency peaks of advertisement calls with bimodal spectra, including those of *H. versicolor*, are typically harmonics of one another, and are probably mechanically constrained to be produced in temporal synchrony, such that there is little scope for independent variation in the fine-temporal characteristics of the two peaks. However, there are other anuran species that produce calls with bimodal, but temporally separated, frequency spectra (Ryan and Rand, 1990). There may be more leeway in such species for independent selection on the temporal characteristics of each frequency component, and it would be interesting to investigate frequency channel-dependent temporal selectivity in such species. Regardless, frequency channel-dependent temporal selectivity may be an important factor in communication in natural chorus environments. Anuran choruses are notably loud and complex acoustic environments, with often high levels of overlap between the calls of conspecifics and heterospecifics (Schwartz and Bee, 2013; Wells and Schwartz, 2007). Studies of the evolutionary consequences of heterospecific noise have focused on the possibility that species partition the acoustic niche by diverging in call frequency (Amézquita et al., 2006; Chek et al., 2003; Drewry and Rand, 1983). Frequency channel-dependent temporal selectivity may be an additional mechanism enabling conspecific mate recognition in dense choruses: signal evaluation would be facilitated if temporal selectivity is greater in the frequency channel that is less affected by heterospecific noise. This possibility has not been investigated, but could be addressed by studies of temporal selectivity in masking noise of different frequencies and bandwidths, and of the distribution of frequency channel-dependent temporal processing strategies in different co-occurring species. Comparative studies would be valuable in determining the extent of evolutionary modification of temporal selectivity in the auditory periphery both across species and among populations within a species exposed to different chorusing conditions.

Not all anuran advertisement calls have a bimodal frequency spectrum (Richards, 2006). Bimodal calls may have originated by

a sensory bias mechanism in which the additional acoustic energy in a second frequency channel rendered such calls automatically more attractive to females because both inner-ear organs were stimulated (Ryan and Rand, 1990). In some versions of the sensory bias hypothesis, any increase in signal complexity that stimulates additional components of the nervous system is predicted to be more attractive (Ryan and Keddy-Hector, 1992). However, sensory systems are also biased against certain patterns (e.g. Gerhardt and Höbel, 2005), and receivers may have biases for characteristics in multiple different stimulus dimensions that are not necessarily concordant. Thus, an additional frequency peak may render calls more attractive in terms of spectral composition, but because females also evaluate temporal signal characteristics, the attractiveness of this peak could depend on its fine temporal structure. Previous studies of female preferences in species that naturally produce calls with a unimodal frequency spectrum found that females prefer unimodal to bimodal calls, which argues against the sensory bias explanation (Bosch and Boyero, 2003; Gerhardt et al., 2007; Witte et al., 2001). The most likely explanation for the reduced attractiveness of calls with a bimodal frequency spectrum in these species is neural inhibition caused directly by the second frequency peak, but our data raise the possibility that the temporal selectivity of the newly stimulated inner-ear organ may also play a role. Male *H. versicolor* advertisement calls have a bimodal frequency spectrum, and females preferred bimodal calls to unimodal calls (Gerhardt, 2005). In two-speaker trials, we found that when one alternative had only one frequency peak (with conspecific AM), a bimodal alternative with conspecific AM on one peak and heterospecific AM on the other was neither more nor less attractive. This indicates that altered AM patterns are not necessarily inhibitory, but neither do they act as super-stimuli, in which the stimulation of the second inner-ear organ automatically results in a more attractive signal. This also goes against an automatic sensory bias explanation and suggests that temporal selectivity in both frequency channels plays a role in determining the attractiveness of calls with bimodal frequency spectra.

Conclusions

We found evidence that temporal selectivity depends on the frequency channel in *H. versicolor*: there was a stronger negative effect on call attractiveness of altering AM in the high-frequency peak than of altering AM in the low-frequency peak. When considered along with some contrasting results from previous studies, it seems that there is considerable variation in the conditions leading to differential temporal selectivity in the two frequency channels of the auditory system. The consequences of this selectivity are poorly understood but there are potentially important implications for both the understanding of the neurophysiological mechanisms of hearing and the evolution of signaling strategies in complex environments. The evolution of complex signals has recently become the subject of intense research. Much of this effort has focused on the functional consequences of complex signaling in terms of benefits to signaler and receiver (Candolin, 2003; Hebets and Papaj, 2005). Less attention has been given to understanding how the nervous system processes and integrates the different types of information contained in complex animal signals. Our demonstration of interactions between the sensory processing of different signal components emphasizes that considering these components in isolation will give an incomplete understanding of the mechanistic and functional bases of communication.

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