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Sensory-motor tuning allows generic features of conspecific acoustic scenes to guide rapid, adaptive, call-timing responses in túngara frogs

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Male frogs court females from within crowded choruses, selecting for mechanisms allowing them to call at favourable times relative to the calls of rivals and background chorus noise. To accomplish this, males must continuously evaluate the fluctuating acoustic scene generated by their competitors for opportune times to call. Túngara frogs produce highly frequency- and amplitude-modulated calls from within dense choruses. We used similarly frequency- and amplitude-modulated playback tones to investigate the sensory basis of their call-timing decisions. Results revealed that different frequencies present throughout this species' call differed in their degree of call inhibition, and that lower-amplitude tones were less inhibitory. Call-timing decisions were then driven by fluctuations in inhibition arising from underlying frequency- and amplitude-modulation patterns, with tone transitions that produced steeper decreases in inhibition having higher probabilities of triggering calls. Interactions between the varied behavioural sensitivities to different conspecific call frequencies revealed here, and the stereotyped amplitude- and frequency-modulation patterns present in this species' calls, can explain previously surprising patterns observed in túngara frog choruses. This highlights the importance of understanding the specific sensory drivers underpinning conspecific signalling interactions, and reveals how sensory systems can mediate the interplay between signal perception and production to facilitate adaptive communication strategies.

1. Introduction

In many frog and insect species, males call to attract females from within dense choruses [1]. Though chorusing can provide benefits such as dilution of individual predation risk and increased *per capita* mating prospects [2,3], the cacophony of rivals' calls poses risks to effective call transmission. Interference from background chorus noise can impede female abilities to recognize and localize calls, and reduce their ability to discriminate in favour of preferred call characteristics [4,5]. Additionally, more specific forms of local interference can also be detrimental; when females hear multiple competitors' calls closely in time their sensory systems often parse these calls such that one call is favoured due to its temporal relationship with the other [6]. Species differ in how acoustic properties vary throughout calls and which call features are crucial for attracting females. This can lead to interspecific variation in which call-timing configurations are salient, and which relative temporal positions are preferred by females. For instance, in many frog and insect species, females exhibit a 'precedence effect', preferring the leading call of an overlapping call pair [7–9]. Conversely, females of certain frog species

discriminate against leading calls of overlapping call pairs, due to attractive features of leading calls being obscured [10,11]. Female preferences for calls at certain relative temporal positions consequently select for male call-timing responses that allow them to preferentially place calls in these preferred positions relative to nearby rivals [6]. Thus, the evolution of male call-timing heuristics is ultimately driven by biases within female perceptual systems [7,12].

Though female perceptual biases are the selective agent shaping male call-timing strategies, these strategies are facilitated proximately by male sensory systems, as males track the fluctuating acoustic scene generated by the calls of nearby rivals for opportune moments to enact beneficial call-timing adjustments [13,14]. As noted above, interspecific variation in call morphologies and female biases can interact to make different relative call-timing positions profitable for males of different species. Similarly, different call morphologies provide males with different acoustic landmarks to use as guides for enacting beneficial call-timing responses relative to rivals' calls. For instance, in many chorusing insects and anurans that produce lengthy continuous (or near-continuous) calls, males remain inhibited from calling from the onset of a rival's call throughout its duration, then call when released from inhibition at the offset of this rival's call [15,16]. Conversely, in anurans that produce pulsatile calls with sufficiently long internote intervals, similar inhibition and release from inhibition dynamics operate on a note-by-note basis throughout the calls of interacting rivals, allowing interdigitation (alternation without overlap) of individual notes within overlapping calls [17]. Finally, some species' calls exhibit complex amplitude [18] and frequency [19] dynamics throughout their duration, providing additional acoustic patterns that can guide call-timing responses. For example, *Mecapoda* katydids produce a complex call consisting of a trill followed by several chirps, and complex timing interdependencies are evident between these distinct elements of the calls of interacting rivals [18]. These examples demonstrate that understanding how males utilize different acoustic landmarks and patterns occurring throughout rivals' calls when making call-timing decisions is crucial for our understanding of how sensory-driven call-timing decisions interact to produce emergent collective chorusing dynamics.

Overall then, it is clear that to understand the evolution of male call-timing strategies we must understand: (i) which temporal relationships with rivals' calls are profitable targets for male call-timing strategies, (ii) the types of adjustments males are capable of making to enact these strategies (e.g. phase advance/delay or gap detection [6]) and (iii) the proximate sensory cues males use as guides for enacting profitable call-timing adjustments. Here, we investigated the acoustic sensory drivers of call-timing decisions in túngara frogs. Amplitude and frequency are highly modulated throughout túngara frog calls (figure 1), and male call-timing decisions are influenced by both of these properties of rivals' calls [20]. Amplitude and frequency modulation occur in parallel throughout calls, meaning that identifying the additive and interactive contributions that these acoustic properties make when influencing call-timing responses requires experimentally decoupling them. To do so, we observed male call-timing responses to playback stimuli consisting of transitions between tones varying in frequency and relative amplitude. Males exhibit a gap-detection strategy in this species; they are inhibited from calling by sufficiently intense acoustic stimulation, and call when they encounter relatively less inhibitory moments ('gaps') within ongoing stimulation [20]. Thus, if a transition from tone A to tone B triggers calls with a high probability, we can deduce that this transition represents a release from inhibition, indicating that males find tone B relatively less inhibitory than tone A [21]. By utilizing tone pairs of many frequency and relative amplitude combinations, we were able to investigate how frequency and amplitude interacted to influence degrees of inhibition and resulting call-timing decisions. Our hypotheses and predictions were as follows:

H1. Transitions from higher-amplitude tones to relatively lower-amplitude tones will induce a release from inhibition.

Frogs are sensitive to the amplitude of acoustic stimulation and find higher amplitudes more inhibitory [21]. Therefore, we predicted that during transitions between tones of equivalent frequency, transitions from higher-amplitude tones to relatively lower-amplitude tones would have higher probabilities of eliciting calls.

H2. Transitions from tones exhibiting more sensitive frequencies to tones exhibiting less sensitive frequencies will induce a release from inhibition.

Túngara frogs differ in their auditory sensitivity to different frequencies [22,23], and we broadly expected that frequencies to which they showed higher sensitivity in neurophysiology studies would be more inhibitory. Thus, we predicted that, when tone amplitude is held constant, transitions from more sensitive to less sensitive frequencies would have higher probabilities of eliciting calls.

H3. The magnitude of the release from inhibition induced by a transition between tones will be influenced by both the relative amplitudes and relative sensitivities of these tones.

We anticipated that both tone frequency and amplitude would influence degree of call inhibition, and that transitions from more inhibitory to less inhibitory tones would have higher probabilities of eliciting calls. Thus, we predicted that the probability that tone transitions elicited calls would depend on interactions between pre- and post-transition tone frequencies, and the relative amplitude difference between them.

H4. Tone transitions sequentially stimulating both auditory end organs will induce a release from inhibition.

In frogs, two auditory organs are responsible for the sensation of frequency [24]. In túngara frogs, lower frequencies (<1500 Hz) present in whines primarily stimulate the amphibian papilla (AP), while higher frequencies (>1500 Hz) present in chucks primarily stimulate the basilar papilla (BP) [23]. As discontinuity in which papilla is being stimulated may be experienced as a release from inhibition, we predicted that tone transitions that cross the AP/BP frequency divide would have higher probabilities of eliciting calls.

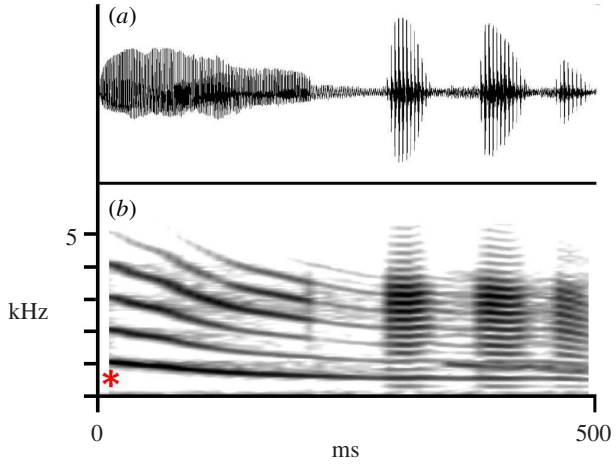

Figure 1. Waveform (*a*) and spectrogram (*b*) of a complex túngara frog call consisting of a whine with three chucks appended. The red asterisk highlights the fundamental frequency of the whine.

2. Methods

(a) Túngara frogs

Túngara frog males float in water to call, with breeding sites typically being shallow pools. Calls consist of an obligatory whine which can be elaborated by appending a number of chuck notes [25; figure 1]. Whines on their own (simple calls) are sufficient to attract females, but whines with chucks appended (complex calls) are fivefold more attractive to females than simple calls [26]. When chorusing with rivals, males typically append 1–3 chucks to their whines [11].

Whines exhibit a continuous descending frequency sweep whose fundamental frequency decreases from ~1000 to ~450 Hz over ~330 ms [27,28; figure 1]. Synthetic whines expressing only the fundamental frequency are sufficient to elicit phonotaxis by females and calling responses by males, and excluding the upper harmonics does not decrease call attractiveness [29]. This suggests that the fundamental is the most salient harmonic present in the whine. Chucks are short (<100 ms) harmonic pulses whose dominant frequency is ~2600 Hz [27]. In anurans, the AP is the auditory organ stimulated by lower frequencies (<1500 Hz) and so is primarily stimulated by whines, while the BP is stimulated by higher frequencies (>1500 Hz) and so is primarily stimulated by chucks [23,24].

Túngara frog males call within dense choruses in which conspecific acoustic interference can reduce the attractiveness of calls [11]. To minimize interference, males employ a gap-detection mechanism whereby calls are triggered when males encounter relatively less inhibitory 'gaps' in the fluctuating acoustic scene at the chorus [20]. Male call-timing responses to rivals' calls are influenced by variation among rivals in the amplitude and frequency trajectories of their calls [20], suggesting male gap-detection mechanisms are sensitive to both amplitude and frequency.

(b) Experimental subjects

In September and October 2023, we collected male frogs as members of amplectant pairs from urban breeding sites around Gamboa, Panama (9°070″N, 79°419″W), near the facilities of the Smithsonian Tropical Research Institute. We separated males from their associated females for trials and, after trials, we weighed males (g), measured their snout-vent-length (SVL; mm) and clipped two hind toes to avoid re-capturing the same individuals. We then reunited pairs and released them back to their capture location later that same night.

(c) Experimental stimuli

To investigate how male call-timing mechanisms responded to amplitude and frequency modulation, we generated many stimuli consisting of two tones; one longer higher-amplitude 'reference tone' within which were interspersed four short 200 ms 'test tone' segments which could vary in amplitude and frequency relative to the reference tone (stimulus exemplars are shown in figure 2). The median interonset interval (IOI: the time elapsing between onsets of successive calls by a caller) for chorusing túngara frogs is ~1.75 s [20] and each stimulus was 2.46 s in duration, meaning males needed to call during stimuli to maintain preferred call rhythms. In túngara frogs, calls are triggered when males experience a release from acoustic inhibition [20]. Thus, if males preferentially call in the short test tone segments of a stimulus rather than reference tone segments, this indicates that the transition from reference tone to test tone represents a tangible release from inhibition, suggesting the test tone is meaningfully less inhibitory than the reference tone.

For tones used to create these stimuli, we chose six frequencies from throughout the túngara frogs' call; five from the typical sweep of the fundamental frequency of the whine (1000, 850, 700, 550 and 400 Hz; as noted, the fundamental is the most salient harmonic of the whine [29]) and one from the frequency range of the chuck (2100 Hz). Though chucks tend to have higher dominant frequencies than this (~2600 Hz: [27]), 2100 Hz maximally stimulates the BP, the auditory organ primarily stimulated by chucks [22,23]. Each stimulus thus consisted of a reference tone of a certain frequency paired with a test tone of

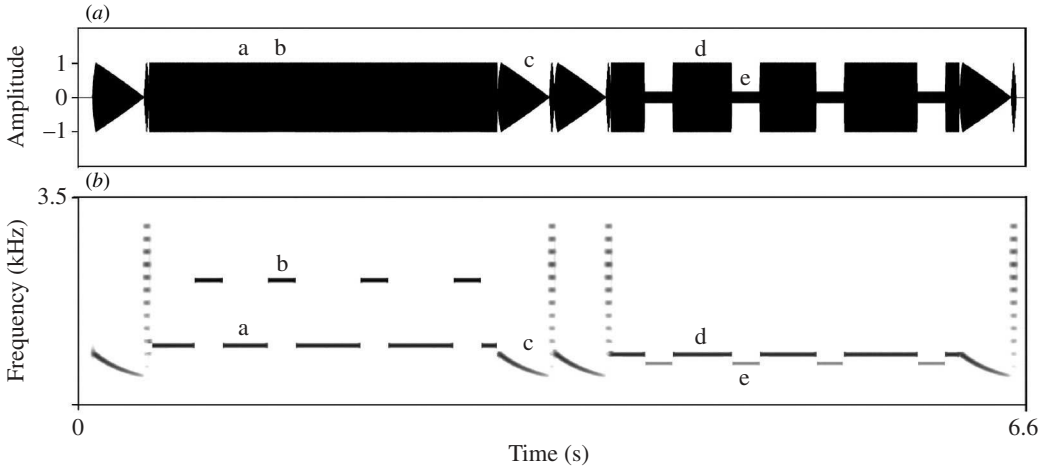

Figure 2. Waveform (*a*) and spectrogram (*b*) illustrations of a section of playback track containing two stimuli. Transitions between reference tone (a, d) and test tone (b, e) segments can be seen within each stimulus. Stimuli are separated by synthetic whine-chucks (c). Each stimulus is 2.46 s in duration, with test tones making up 33% of the duration.

a certain frequency, and we generated all possible pair-wise combinations of our chosen frequencies, including self-pairings. Additionally, for each frequency pairing, we varied the amplitude of the test tone relative to the reference tone. Reference tones were always broadcast at 82 dB (sound pressure level; re. 20 µPa) at the male's location, while test tones were broadcast at amplitudes ranging from 100% to 16% the amplitude of reference tones, in 7% increments. Thus, test tone amplitudes ranged from 82 to 66.1 dB at the male's location (a 6 dB decrease in SPL represents a 50% reduction in amplitude). This yielded 13 possible amplitude relationships between reference and test tones for each of the 36 possible frequency pairings, resulting in 468 unique stimulus combinations.

When generating the above stimuli, we minimized nonlinearities produced by abrupt tone onsets and offsets by beginning and ending stimuli at the point where tone sinewaves crossed zero, and joining sequential tone segments at points where both sinewaves crossed zero [30]. Additionally, we included 3 ms linear amplitude ramps at stimulus onsets and offsets, and 3 ms ramps between reference tones and lower-amplitude test tone segments within stimuli. As males undergo intercall changes in responsiveness to call triggers [20,31], we spaced test tone segments approximately evenly across each stimulus to ensure at least one test tone segment per stimulus would be encountered at the appropriate time to potentially trigger a call. However, we also introduced some random temporal variation. Thus, successive 200 ms test tone segments within stimuli were separated by reference tone segments of 430 ms ± 60 ms (mean ± s.d.) duration. Onset of the first test tone segment never occurred earlier than 200 ms into the reference tone of the stimulus.

(d) Playback trials

For playback trials, we presented stimuli to males in unique randomized orders. During playback, successive stimuli were separated by two or three back-to-back synthetic whine-chuck calls (with no intervening silence) corresponding to 0.7–1 s elapsing between successive stimuli (figure 2). The number (two or three) of intervening calls was chosen randomly to produce heterogeneity in the temporal spacing of successive stimuli. Additionally, these intervening calls provided stimulation for males to keep calling throughout our lengthy playback sessions (~27 min total duration). We conducted all playback trials in a hemi-anechoic chamber (Acoustic Systems; ETSLindgren, Austin, TX, USA). During trials, males were contained in a 5 cm diameter acoustically transparent enclosure containing ~2.5 cm deep water in which they could float to call (water temperature ranged from 27.2 to 29°C). This enclosure was located 1.35 m away from the playback speaker. We desired reference tones of all frequencies to register at 82 dB at the male's location. Thus, to accommodate the frequency response curve of the playback speaker, we split the main stimulus track into six separate tracks, each containing tones of a single frequency. We then separately adjusted the relative amplitudes of these tracks upstream of the speaker, such that maximum-amplitude tones of each frequency registered at 82 dB SPL at the male's location. Prior to each trial, we encouraged males to call via playback of a synthetic whine-chuck playing every 1.75 s. We allowed males to call consistently with this for 1 min, then commenced playback. Not all males called throughout the full playback duration, and we ended trials early if males ceased calling for several minutes. We recorded all calls produced by subjects during playback onto a Zoom F6 recorder via a Synco LavS6R tie-clip microphone. We used tools from the Librosa Python package [32] to extract precise timestamps for the onsets of all calls produced during playback, and visually verified their accuracy prior to analysis. In total, 43 males produced 23 168 calls that occurred during our tone stimuli.

(e) Analysis

We cross-referenced observed call onset timestamps with known timestamps of stimulus onsets, and onsets and offsets of reference and test tone segments within stimuli (see electronic supplementary material, S1 for a cautionary note regarding

cross-referencing in this way). This revealed the stimulus during which each call onset occurred, and whether it occurred during the reference tone or test tone. We excluded calls beginning within the first 70 ms of a stimulus because males of this species exhibit an effector delay (the time elapsing between a call being triggered and actually being produced) of ~50 ms [33]. This means that calls occurring this early into the stimulus were almost certainly initiated in the nervous system prior to stimulus onset. Counting such calls as being indicative of a male deciding to call over the initial reference tone segment of that stimulus could thus produce erroneous datapoints. Though the potential for such errors is also present at all internal reference/test tone transitions, the onset of each stimulus is immediately preceded by the end of an interstimulus whine-chuck. Males readily call over these whine ends [11], suggesting stimulus onsets are at heightened risk of such errors. The proportion of stimulus durations (excluding the first 70 ms) comprised by test tones is 0.33; this then represents the expected probability that a male calling randomly during a stimulus would place his call in a test tone by chance alone.

To investigate how frequency and amplitude combinations within stimuli influenced whether males preferentially called during test tones, we built a mixed-effects logistic regression model using the 'lme4' R package [34]. As our binary response variable, we included whether each call onset that occurred during a stimulus occurred during the test tone or reference tone of that stimulus (n = 23 168). To investigate how frequency and amplitude differences between reference and test tones influenced the probability that transitions to test tones elicited calls, we included reference tone frequency, test tone frequency and the amplitude of the test tone relative to the reference tone as fixed effects. We also included a three-way interaction between these variables, to investigate possible context dependence. Residuals plots revealed that reference tone frequency and test tone frequency exhibited nonlinear relationships with the log odds of our response variable, so we modelled these variables with natural splines with 3 d.f. This greatly improved model fit (likelihood ratio test: $p < 0.00001$). As additional covariates, we included male mass and water temperature, to control for possible phenotypic and environmental influences on male calling responses, respectively. To control for changes in responsiveness that occur throughout callers' intercall period [20,31], we also included the duration of the IOI that preceded each call and, to control for potential fatigue or habituation effects that might accrue throughout our lengthy playback sessions, we also included the absolute onset times of calls. We standardized all predictor values ((x-mean(x))/s.d.(x)) prior to analysis, with preceding IOI being standardized within males to account for intermale variation in preferred call rates and changes in responsiveness throughout IOIs [20]. To minimize risks of type 1 errors (falsely significant results), we did not perform simplification of the fixed-effect structure of the model [35], and we present full model results in the electronic supplementary material (S2). Additionally, we fit the maximal random-effects structure supported by the data [35]. We included a random intercept for male identity nested within testing night, and initially included correlated random slopes for all predictor variables (except mass and water temperature for which males only experienced a single value). However, when correlated random slopes for certain variables were not supported, we removed correlation terms, then removed random slopes entirely if they remained unsupported. Random-effects summaries are presented in electronic supplementary material, S3.

3. Results

(a) Call-timing rhythms during playback trials

Males called readily during our playback stimuli, with 23 168 calls out of 31 029 total calls (75%) occurring during stimuli. The rest occurred during interstimulus whine-chucks. Onsets of calls occurring during test tones were tightly clustered shortly after test tone segment onsets (figure 3*a*). This suggests that these calls were triggered by the sudden release from inhibition accompanying transitions from relatively more inhibitory reference tones to less inhibitory test tones. The lower limit of the peak of delays between test tone segment onsets and call onsets was ~50 ms, which approximates the effector delay reported for this species by Greenfield & Rand [33].

The median IOI here was 1.73 s (figure 3*b*), which is within the range of median IOIs exhibited by males calling in live choruses of various sizes (1.71–1.74 s, from: [11]). The modal binned (50 ms bins) IOI was between 1.28 and 1.33 s, much shorter than typical IOIs seen in live choruses (see comparison in figure 3*b*). This likely arose due to the temporal patterns of modulation present in our stimuli; males often called twice during a 2.46 s duration stimulus, and 1.3 s corresponds to the typical time elapsing between onsets of 1st and 3rd, and 2nd and 4th, test tone segments within a stimulus. The median coefficient of variation (CV) for IOIs exhibited by males here (0.31) was higher than the median CV observed for males calling in six-male choruses (0.13, from: [11]). This suggests that males responded to the extreme amplitude- and frequency-modulation patterns in our playback by increasing the dynamism of their call rhythms.

(b) Amplitude modulation influenced male call-timing responses

First, to investigate how transitions between tones of different amplitudes without associated frequency changes influenced the propensity for males to call in test tones (H1), we compared the predicted probabilities from our model that males called in test tones for stimuli in which reference and test tone frequencies were identical, but relative test tone amplitudes varied (figure 4*a*). Due to our inclusion of spline terms and interaction terms, the model output is extensive. Thus, we only present results regarding terms of interest in-text, though full model results are presented in electronic supplementary material, S2, and model code is available in the data repository. Throughout the results, when presenting predicted probabilities from our model we present the marginal effects, with all covariates other than those considered held at their mean values. As shown in figure 4*a*,

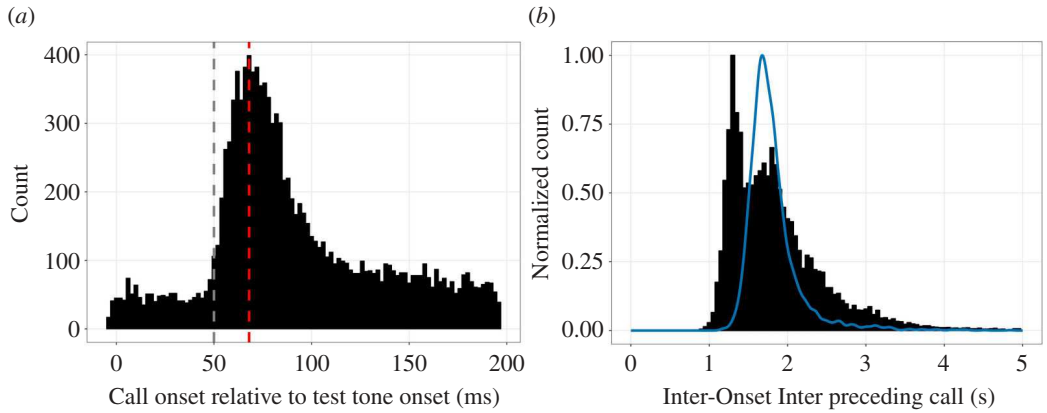

Figure 3. (*a*) Histogram of delays between test tone onsets and call onsets for calls that occurred during test tones (2 ms bins), corrected for sound travel time from playback speaker to frog (1.35 m at 27°C = 3.9 ms). Grey dashed line indicates the 50 ms effector delay described by Greenfield & Rand [33]. Red dashed line denotes modal binned delay (67–69 ms). (*b*) Normalized histogram of IOIs observed in the current study (50 ms bins, $n = 23\,168$). The overlayed blue density plot shows the distribution of IOIs from males interacting in six six-male choruses ($n = 3600$), from Larter & Ryan [11].

for almost all frequencies the probability that males called in test tones increased with decreasing test tone amplitude, though the magnitude and shape of this decrease varied somewhat. The exception was 2100 Hz, with males having similar near-chance probabilities of calling in reference and test tones at all relative amplitudes (chance probability = 0.33).

(c) Frequency modulation influenced male call-timing responses

Then, to investigate how transitions between different frequencies without associated changes in amplitude influenced propensity to call in test tones (H2), we compared the predicted probabilities that males called in test tones for stimuli consisting of all possible frequency pairings but in which reference and test tones were broadcast at identical amplitudes; both 82 dB. With amplitude being identical, any propensity to call in a test tone of a certain frequency when it is embedded within a reference tone of a certain frequency was presumably driven by that test tone frequency being less inhibitory than that reference tone frequency. Results are presented in figure 4*b*. We found that 2100 Hz was the least inhibitory frequency overall, with test tones of 2100 Hz having high, significantly above-chance (probability > 0.33), probabilities of being called in when embedded within any other reference frequency. For frequencies from within the whine range, degree of inhibition increased with increasing frequency; in all cases, relatively lower-frequency test tones were called in at significantly above-chance levels when embedded in higher-frequency reference tones. Thus, all frequencies from our set could be unambiguously linearly ranked from least to most inhibitory (depicted in figure 5). Additionally, as we had predicted that frequencies to which túngara frogs have higher auditory sensitivities would be more inhibitory (H2), we also plot sensitivity ranks for these same frequencies derived from auditory tuning curves presented in other studies (figure 5).

Above, we interpreted higher response probabilities during transitions from higher to lower whine frequencies as indicating a positive correlation between whine frequency and inhibition rank. However, as túngara frog whines always decrease in frequency throughout, this could alternatively represent males preferentially responding to stimuli adhering to the natural descending whine pattern [37]. To rule this out, post hoc we analysed responses to a subset of stimuli in which reference and test tones were identical in frequency and amplitude, thus representing 2.46 s duration unmodulated 82 dB tones of a single frequency. Males should have lower probabilities of calling at all during tones of more inhibitory frequencies [38], allowing us an alternative way to assess frequency inhibition rankings. A mixed-effects logistic regression model (details in electronic supplementary material, S4) revealed near-identical frequency inhibition rankings as above (figure 5), though results were less certain due to the smaller sample size (n = 224). Males had the highest probabilities of calling during 2100 Hz tones and, for whine frequencies, had progressively lower probabilities of calling during tones as frequency increased (frequency, predicted probability males called during tone (95% CI for prediction): 2100 Hz, 0.99 (0.89, 1); 400 Hz, 0.91 (0.76, 0.97); 550 Hz, 0.79 (0.65, 0.89); 700 Hz, 0.69 (0.53, 0.81); 850 Hz, 0.63 (0.49, 0.76) and 1000 Hz, 0.63 (0.44, 0.78); electronic supplementary material, figure S4). Thus, the patterns in figures 4*b* and 5 are adequately explained by frequencies differing in their degrees of inhibition.

(d) Amplitude and frequency modulation interacted to influence male call-timing responses

The marginal effects for our significant ($p < 0.001$) three-way interaction between reference tone frequency, test tone frequency and relative test tone amplitude are visualized in figure 6 (H3). The same relative rankings of tone frequencies shown in figure 5 were evident, as was the increase in probability of calling in test tones as test tone amplitudes decreased. However, the probability that a transition to a test tone of a given frequency and amplitude elicited a call was strongly influenced by the frequency of the reference tone directly preceding it. This dependence on immediate acoustic context can be seen by comparing the intercepts and shapes of similarly coloured curves (depicting the influence of relative test tone amplitude on the probability that a test tone of a given frequency was called in) across different subfigures (denoting different reference frequencies that test tones were embedded within).

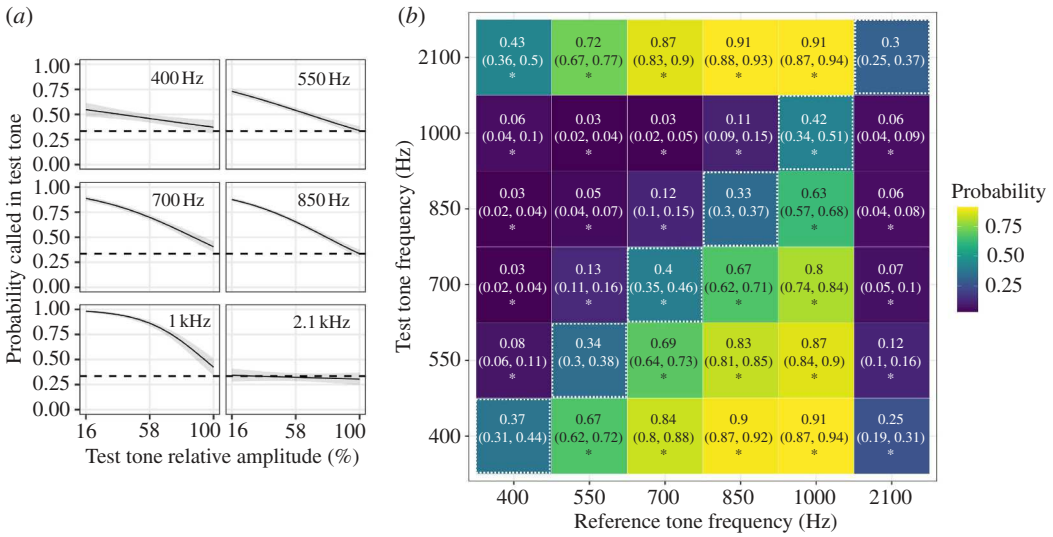

Figure 4. (*a*) Predicted probabilities (and 95% CIs for predictions) that calls occurred during test tones rather than reference tones for stimuli in which reference and test tones were of the same frequency (noted in top-right of subfigures) across a range of relative test tone amplitudes (16–100% amplitude of reference tones). The dotted line at 0.33 represents the expected probability that males would call in test tones by chance alone. (*b*) A heat map representing the predicted probabilities (and 95% CIs for predictions, in parentheses) that calls occurred during test tones rather than reference tones for stimuli consisting of different test tone frequency (*y*-axis) and reference tone frequency (*x*-axis) pairings, in which test tones and reference tones were broadcast at identical amplitudes (82 dB). Heat map colours are scaled to these probabilities (see legend). Asterisks (*) denote significantly different-than-chance probabilities (0.33 not contained in 95% CI). White hatching around diagonal cells indicates self-pairings.

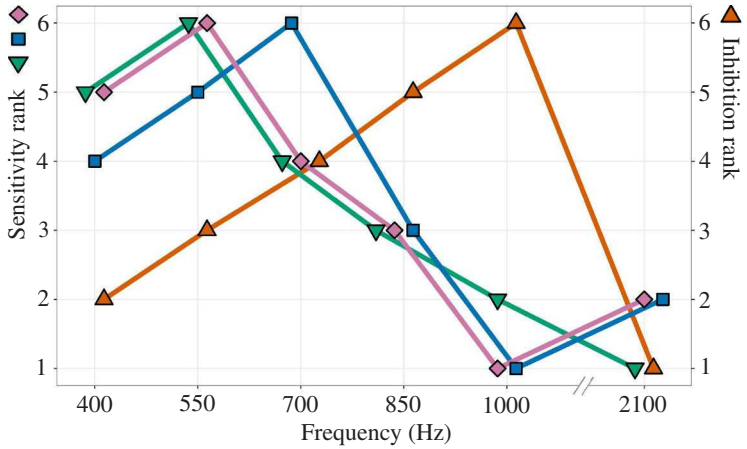

Figure 5. Inhibition rankings of frequencies used in this study when presented at the same amplitude (82 dB) (orange triangles; 6 = most inhibitory and 1 = least), as well as rankings of sensitivities to these same frequencies as reported in previous neurophysiological studies, or as inferred from reported trends (green downward triangles from Ponnath *et al.* [22]; blue squares from Ryan *et al.* [23]; pink diamonds from Wilczynski *et al.* [36]). For sensitivity rankings, 6 = most sensitive (lowest auditory threshold) and 1 = least sensitive (highest auditory threshold). Points have been connected to produce inhibition/sensitivity functions. Some horizontal jitter has been added to avoid overlapping points/lines. See electronic supplementary material, S6, for a similar plot of inhibition ranks against reported auditory brainstem response sensitivities.

Most of our control covariates had only negligible, non-significant, effects on male propensities to call in test tones (see electronic supplementary material, S2, for all fixed-effects estimates; see electronic supplementary material, S5, for visualizations). However, the onset time of calls within our playback trials had a significant effect ($p < 0.001$), though a small one, with the probability that males called in test tones increasing by 2–3% over the course of the 27 min playback trial. This suggests that males underwent some minor degree of acclimation to the acoustic playback environment. Our initially complex random-effect structure had to be simplified due to convergence issues (see electronic supplementary material, S3, for final random-effects structure and estimates), and the remaining random effects in our model explained little variance in our outcome variable, as indicated by the conditional R^2 being negligibly larger than the marginal R^2 (marginal R^2: 0.55; conditional R^2: 0.56). This suggests that the tuning of gap-detection mechanisms is not subject to much intermale variation.

4. Discussion

We investigated how frequency and amplitude modulation within tonal stimuli influenced male call-timing decisions in a gap-detecting frog species with amplitude- and frequency-modulated calls, the túngara frog. Our results illuminate how

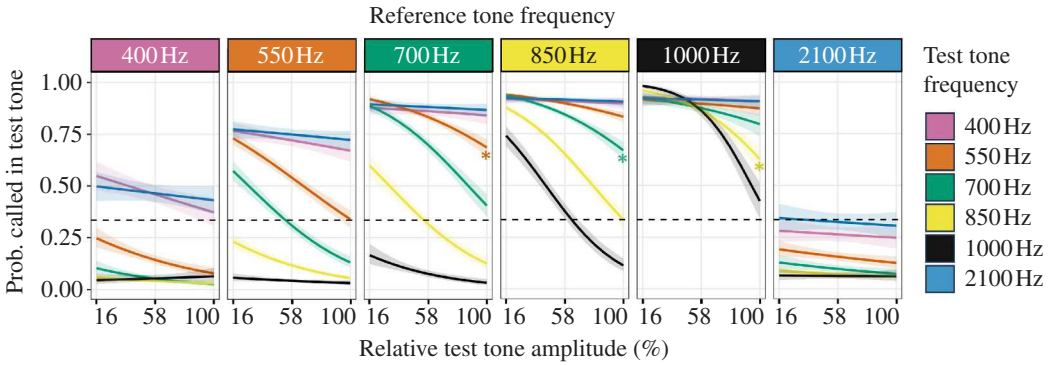

Figure 6. A visualization of the three-way interaction between reference tone frequency, test tone frequency and relative test tone amplitude, in predicting the probabilities that transitions from reference to test tones triggered calls. On the *y*-axis is the probability that a call occurring during a stimulus began in a test tone segment rather than the reference tone, and on the *x*-axis is the amplitude of the test tone relative to the reference tone. Each subplot represents stimuli with reference tones of a certain frequency (indicated at the top of the subfigure), and lines within each subplot represent the relationship between relative test tone amplitude and the probability that calls occurred in test tones of a certain frequency (indicated by line colour; see legend on the right) when embedded in that reference tone. A horizontal dashed black line has been added at 0.33 (the expected probability that a call would occur in a test tone by chance alone). Asterisks (*) denote reference/test tone frequency transitions mentioned later in the discussion.

males' call-timing mechanisms parse the fluctuating acoustic scene generated by their competitors, and contribute to a fuller understanding of the sensory drivers of the call-timing decisions from which consequential collective chorusing patterns then emerge. Túngara frogs share many details of their communication ecology with other chorusing frogs and insects [13], and similar signal coordination strategies are evident in other acoustically signalling taxa such as birds [39] and mammals [40]. Thus, the insights generated here have general importance for understanding the interplay between signal perception and production and the evolution of acoustic communication strategies in social species.

(a) Frequency and amplitude modulation influenced call-timing decisions

When investigating how inhibited callers were by tones of different frequencies present throughout this species' calls, we found that the degree of call inhibition differed by frequency. The least inhibitory of our chosen frequencies was the exemplar from within the chuck range (2100 Hz), which showed a near-complete lack of call inhibition at all amplitudes tested (figures 4 and 5, electronic supplementary material, S4). This is the only frequency in our set that stimulates the BP (the anuran auditory organ stimulated by frequencies >1500 Hz) rather than the AP (stimulated by <1500 Hz). As in other anurans [24], in túngara frogs the BP is less sensitive than the AP, so this result aligns with our predictions that frequencies to which túngara frogs have been demonstrated to be less sensitive in neurophysiology studies would be less inhibitory (H2, figure 5). For frequencies from throughout the whine, which stimulate the AP, we found a perfectly linear positive relationship between inhibition ranking and frequency. This contrasts with what might be predicted based on neurophysiological auditory tuning sensitivities, which suggest a nonlinear relationship between frequency and sensitivity for this frequency set (figure 5, and references in caption). Disagreement between the behavioural salience of frequencies revealed here and their threshold sensitivities highlights that caution is warranted when using threshold sensitivities to infer the functional consequences of auditory system tuning at ecologically relevant supra-threshold stimulation levels [1].

Potentially biologically salient frequency-transition patterns had no obvious effects on male responses. Reference to test tone transitions crossing the AP/BP frequency divide did not generate calling responses that differed in kind from transitions entirely within the AP range (H4). Though 2100 Hz test tones (our BP range exemplar frequency) had high probabilities of being called in when embedded within reference tones of all AP frequencies, as the BP is the less sensitive auditory organ and 2100 Hz was correspondingly the least inhibitory frequency (figure 4, electronic supplementary material, S4), this simply fits within the broader observed pattern in which transitions from more inhibitory reference tones to relatively less inhibitory test tones had high probabilities of triggering calls. Similarly, though eliciting female phonotaxis in this species requires stimuli to approximate the downward frequency sweep of the whine by beginning at frequencies above 560–600 Hz and transitioning to frequencies below this threshold [22,28], there was no evidence for increased male response probabilities to transitions crossing this threshold. For example, in figure 6 the curves depicting transitions from 700 Hz reference tones to 550 Hz test tones (which cross this salient frequency divide) and transitions from 850 Hz reference tones to 700 Hz test tones (which do not) are very similar (curves highlighted in figure 6 by orange and green asterisks, respectively). Additionally, males did not seem to preferentially respond to transitions broadly adhering to the decreasing frequency patterns present in whines, as response patterns could be adequately explained simply by the relative inhibition ranks of different frequency pairings (figure 4*b*; electronic supplementary material, S4). This lack of selectivity, and that call response latencies to test tone segment onsets are sufficiently rapid (~68 ms; figure 3*a*) as to likely preclude any higher forebrain call recognition [41,42], suggest that male call-timing decisions do not involve higher-level processing of species-specific temporal frequency patterns. Rather, males seem to track more generic features of conspecific acoustic scenes via patterns of differential inhibition by different conspecific frequencies. Male túngara frogs do show some selectivity for species-specific call patterns when performing phonotaxis towards conspecific calls [43] and when being stimulated to begin calling [37], suggesting different neural circuits may underpin these slower preparatory responses and the rapid call-timing decisions made once calling has been initiated [41].

As has been shown in other frog species [21], amplitude modulation also had important influences on call-timing decisions; for almost all test and reference tone frequency combinations, transitions to test tones had higher probabilities of eliciting calls as test tone amplitude decreased (H1; figure 4). Thus, the degree of call inhibition induced by acoustic stimulation is a function of both frequency and amplitude (H3). This species' call begins with a whine that decreases from high to low frequency within the AP range and then culminates in BP-stimulating chuck notes. This means that, as calls progress, the frequencies they contain become progressively less inhibitory when broadcast at identical amplitudes (82 dB). However, calls are also highly amplitude modulated, and amplitude and frequency modulation are inextricably coupled. Amplitude and frequency decrease in parallel throughout whines, likely combining to produce a steep decrease throughout whines in the degree of inhibition induced in listening rivals, the consequences of which we discuss later. Then terminal chucks are the highest frequency and usually highest amplitude part of the call [25,27]. In fact, chucks can be upwards of twice the peak amplitude of whines, suggesting that the relative lack of inhibition induced by our chuck exemplar frequency here may be an artefact of our capping all frequencies at the same amplitude.

One of our most striking findings was that the probability that a transition to a test tone of a given frequency and amplitude combination elicited a call was highly context dependent (H3; figure 6). Transitions to certain test tones varied in whether they were called in at above-chance or below-chance levels depending on the reference tone in which they were embedded. Though, given the unambiguous inhibition rankings of frequencies used in this study (figure 5), this is simply what would be expected if the relative rankings of test and reference tone frequencies switched sign across certain frequency pairings. However, even when transitions to a given test tone frequency elicited calls at above-chance levels when preceded by multiple different reference tone frequencies, these probabilities differed depending on reference tone frequency (figure 6). Thus, it was not simply the properties of test tones themselves that determined the probability that encountering them within ongoing acoustic stimulation elicited calls, but rather how the properties of test tones related to the properties of the reference tones directly preceding them. This dependence on immediate acoustic context hints at certain properties of male call-timing mechanisms, which we explore below.

(b) Clues regarding the túngara frog call-timing mechanism

In frogs, avoidance of call overlap is the norm [44] and males of many species exhibit gap-detection strategies in which they remain inhibited from calling throughout rivals' calls and then produce their call shortly after the offset of this rival's call. Thus, triggers for calls are typically stated as rival call offsets, or simply as reductions in background noise levels [15,44]. In more crowded choruses with extensive call overlap, rivals' call offsets are often embedded within an ongoing continuous stream of chorus noise, meaning offsets to true silence are rare [11]. Here, when considering call offsets to be call triggers, researchers often invoke 'selective attention' mechanisms by which chorusing males narrowly attend to the calls of a certain subset of salient rivals within earshot [13,33,45]. Thus, call offsets are still invoked as call triggers in crowded and noisy choruses, though only the offsets of these categorically attended-to rivals.

However, observations of túngara frog choruses seemed at odds with males selectively attending to a few rivals while ignoring the rest, and were better explained by males tracking perceived fluctuations in the broader acoustic scene at the chorus. For instance, male call-timing decisions were influenced by emergent features of the acoustic scene not contained within the behaviour of any single rival, such as amplitude spikes arising due to synchronous calling by several nearby rivals [20]. Our current results emphasize that a broader conception of what constitute call triggers in túngara frogs is also helpful; rather than rivals' call offsets *per se* being the triggers for calls, call triggers seem better defined more generically as a sudden sharp decrease in acoustic inhibition (Δinhibition) of a sufficient magnitude, with inhibition being a function of both the amplitude and frequency of acoustic stimulation. Such call triggers will often be rivals' call offsets, but not always. Furthermore, larger Δinhibition values appear to have higher probabilities of triggering a call, all else being equal. This can explain why transitions to the same test tone (of a given frequency and amplitude) had higher probabilities of eliciting calls when preceded by certain reference tone frequencies (figure 6). ΔInhibition is a dynamic variable arising from the juxtaposition of acoustic stimulation experienced at successive timepoints. Thus, for a given test tone, the Δinhibition accompanying a transition from a much more inhibitory reference tone is more drastic than that accompanying the transition from only a somewhat more inhibitory reference tone, yielding a correspondingly higher probability of triggering a call.

Male calling rhythms during playback also illuminate aspects of this species' call-timing mechanism. The median IOI of males interacting in choruses [11], and males responding to playback in the current study (figure 3*b*), was ~1.75 s, suggesting variation in IOIs occurs around this preferred call rate. However, the modal IOI here was ~1.3 s, and the minimum was 0.8 s. Males never show IOIs of 0.8 s, and seldom show IOIs of 1.3 s, when chorusing with rivals [11,20] (figure 3*b*). The shorter modal IOI here can be explained by a feature of our stimuli: males often called twice during a 2.46 s duration stimulus, and 1.3 s corresponds to the typical amount of time elapsing between onsets of the 1st and 3rd, and 2nd and 4th, test tone segments within a stimulus. This suggests that males can be induced to call earlier than usual when they experience sufficiently large Δinhibition values, such as those present in some of our playback stimuli.

Narins [31] observed that *Eleutherodactylus coqui* and *Dendropsophus ebraccattus* males showed changes in their probabilities of responding to (calling immediately following) playback calls throughout their IOIs. Males exhibited an absolute behavioural refractory period (ABRP) after they called during which playback never elicited a response. Playback calls encountered immediately following the ABRP had low (but non-zero) response probabilities, but response probabilities progressively increased as playback calls were encountered later into males' IOIs. A similar pattern seems apparent in túngara frogs. That males here never called in two successive test tones in a stimulus (corresponding to IOIs of ~0.65 s) demonstrates the presence

of an ABRP and, previously, chorusing males were shown to become more permissive regarding call triggers as IOIs progressed [20]. Additionally, that the drastic modulations (and drastic Δinhibition values) present in our stimuli could induce males to call much earlier than usual suggests that sufficiently large Δinhibition values have sufficiently high response probabilities to overcome the lower baseline response probabilities occurring earlier during males' IOIs. Thus, response probabilities gradually increase throughout IOIs but, at any given point, are also weighted by the magnitude of Δinhibition values encountered.

Taken together, the functioning of the túngara frog call-timing mechanism seems to resemble the following:

- Calls are triggered by a steep decrease in inhibition by acoustic stimulation (Δinhibition) of sufficient magnitude, with larger decreases having higher probabilities of triggering an imminent call (higher response probabilities), all else being equal;
- Immediately after calling, males exhibit an absolute behavioural refractory period (ABRP) [31] during which they are unable to call (response probabilities are ~0);
- Shortly after the ABRP elapses (minimum IOI here: 0.8 s), response probabilities are low, but non-zero. However, sufficiently large Δinhibition values can overcome these initial lower baseline response probabilities to trigger calls earlier than is typical. Response probabilities increase steadily throughout IOIs, meaning that Δinhibition values of progressively lower magnitudes become sufficient to elicit calls as IOIs progress, i.e. males become progressively more permissive regarding call triggers; and
- Eventually, if no appropriate call triggers (sufficiently large Δinhibition values) are encountered, calls are triggered endogenously regardless of current acoustic stimulation [20].

(c) Simple mechanisms can produce complex adaptive patterns in interaction with varied acoustic environments

This mechanism offers an elegant solution for many challenges faced by frogs calling in noisy choruses. An attractive property of Δinhibition as a call trigger (as opposed to attended-to rivals' call offsets, narrowly) is that it is a generic outcome of fluctuations in the properties of acoustic stimulation that requires no higher forebrain call recognition. This allows rapid responses (~68 ms) to rivals' calls and fluctuations in ongoing chorus noise [41,42]. Another attractive property is that because Δinhibition arises from relative comparisons of successive acoustic moments, if inhibition decreases sufficiently from one moment to the next a call will be triggered. Thus, flexibility across acoustic environments is 'baked-in', and males will tend to call during local inhibition minima regardless of how absolutely inhibitory these minima are. This is observable in figure 6 in the similar response probability curves for different tone pairings that differ in how inhibitory corresponding reference and test tones are across pairings (figure 4*b*); compare curves for 1000 to 850, 850 to 700 and 700 to 550 Hz (curves highlighted in figure 6 with orange, green and yellow asterisks, respectively). Finally, that response probabilities gradually increase throughout IOIs, while also being weighted by the magnitude of the call trigger (Δinhibition) encountered, means males will typically call at preferred rates but can produce earlier calls to capitalize on conspicuous and unpredictable short-lived lulls in chorus noise when they appear. Furthermore, the most pronounced Δinhibition values encountered by a male will arise from the call offsets of his nearest rivals' calls to silence (in smaller choruses) or to low-amplitude distant chorus noise (in larger ones). These will produce correspondingly high probabilities of triggering a following call, even when encountered relatively early during a male's IOI, thus allowing males to align their call rhythms with their nearest neighbours' rhythms to preferentially facilitate alternation without overlap with these salient competitors.

Túngara frog sensory systems are tuned to most strongly represent conspecific call frequencies (see 'Matched Filter Hypothesis' in: [23,46]). This means that, though Δinhibition as a call trigger does not require call recognition *per se*, conspecific calls would still be prioritized during call-timing decisions over other noise types because conspecific frequencies produce the most pronounced inhibition peaks within frogs' perception. Furthermore, differential inhibition by different frequencies occurring throughout conspecific calls would allow Δinhibition as a call trigger to reliably produce specific beneficial call-timing relationships with conspecific rivals' calls, by interacting with the predictable, stereotyped, frequency and amplitude progressions present throughout túngara frog calls [27]. Similarly, frog choruses show emergent regularities due to species-specific call properties, rhythms and calling interactions [47], and the interaction between the aforementioned call-timing mechanism and features of acoustic scenes generated in different túngara frog chorusing environments can explain flexibility in interaction patterns observed across different social environments, as we explain below.

In smaller túngara frog choruses (≤3 males) males alternate calls without overlapping, presumably because the offsets of nearby rivals' calls to silence are the most conspicuous Δinhibition values encountered, with high probabilities of triggering following calls. However, beyond this chorus size threshold, highly stereotyped call overlap becomes increasingly prevalent [11,20]. During said overlap, following callers' call onsets almost invariably occur at the tail end of leading callers' whines, just before the chucks. This configuration causes overlapped leading calls to be discriminated against, thus favouring following calls [11]. Whines decrease in amplitude as they progress while, simultaneously, the frequencies they contain become progressively less inhibitory (figures 4*b* and 5). These parallel shifts would combine to produce steep decreases in inhibition (large Δinhibition values) in listening rivals as whines progress (figure 4). As choruses grow larger call overlap becomes increasingly unavoidable and chorus noise becomes more constant, meaning call offsets to true silence become increasingly rare [11]. Consequently, Δinhibition values throughout rivals' whines increasingly become the most salient and commonly encountered potential call triggers, thereby increasingly eliciting these stereotyped overlapping calls. Similar logic can also explain differentiated interaction patterns observed. Males who produced calls with whines which ended at lower amplitudes, and which started at higher frequencies and ended at lower frequencies, had higher probabilities of being overlapped by their chorus mates [20], presumably because these features result in larger Δinhibition values throughout whines. Such density-dependent shifts

in interaction patterns, and differentiated responses among nearby chorus mates, might be tempting to ascribe to cognitively sophisticated flexible calling strategies. However, they can plausibly be explained simply by interactions between the tuning of males' call-timing mechanisms, the stereotyped frequency- and amplitude-modulation patterns of conspecific calls, and emergent regularities in the acoustic scenes generated in different social environments.

5. Conclusions

Túngara frogs produce highly amplitude- and frequency-modulated calls and, by experimentally decoupling these different candidate sensory drivers of call-timing decisions, we were able to demonstrate that call-timing decisions were driven by fluctuations in inhibition that arise as a function of underlying fluctuations in the amplitude and frequency of acoustic stimulation. We incorporated these and previous findings into a novel verbal model of the túngara frog call-timing mechanism. Though certain inferences require experimental validation and there are surely additional details to discover, our model generates plausible explanations for our experimental results and for several previously surprising observations made while studying chorusing interactions. Our explanatory framework emphasizes that adaptive flexibility in calling behaviour across varied social environments need not be underpinned by complex behavioural rules or strategies. Rather, flexibility in chorusing interactions can plausibly arise through simple call-timing mechanisms interacting in complex ways with the frequency- and amplitude-modulation patterns present in conspecific calls, and with the emergent fluctuation patterns generated in different social environments.

Ethics. Research permitted by the Government of Panama (SE/A-39-2020; ARG-078-2022), and procedures approved by STRI-ACUC (SI-21012) and UT Austin IACUC (AUP-2022-00012). All protocols followed the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research [48].

Data accessibility. Data and code are available in Dryad [49].

Supplementary material is available online [50].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. L.C.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, validation, visualization, writing—original draft, writing—review and editing; M.J.R.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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