

The sound of a blood meal: Acoustic ecology of frog-biting midges (*Corethrella*) in lowland Pacific Costa Rica

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Abstract

Animal communication systems are often exploited by eavesdropping antagonists, especially predators and ectoparasites. Female frog-biting midges (Diptera: Corethrellidae) are known to use male anuran advertisement calls to locate their blood hosts, frogs. Here, we use acoustic midge traps broadcasting synthetic and recorded calls to identify those frog call parameters that affect midge attraction. At our study site in Pacific lowland Costa Rica, we found that overall midge attraction was influenced by both spectral and temporal acoustic parameters. Low dominant frequencies (below 1 kHz) and short pulse durations (between 125 and 500 ms) attracted the highest numbers of midges in tests with synthetic sinusoidal pure tones. These preferences partially explained the relative attractiveness of the advertisement calls of ten local frog species. The advertisement calls of the common and widespread Giant Bullfrog, *Leptodactylus savagei* (Anura: Leptodactylidae), attracted by far the largest number of midges, suggesting that this frog species might be a key resource for frog-biting midges in Costa Rica. Although we found that calls of different frog species attracted different midge species/morphospecies in statistically different proportions, acoustic niche differentiation among midge species appeared to be moderate, suggesting either a limited degree of host specialization or the use of additional short-range, that is, chemical, host recognition cues.

KEYWORDS

corethrellidae, eavesdropping, hematophagy, host choice, parasite, phonotaxis

1 | INTRODUCTION

Signals used in animal communication systems are often exploited by illegitimate receivers, such as eavesdropping predators and parasites (Peake, 2005). Eavesdropping antagonists are known to influence the evolution of signals and signaling behavior and senders as well as receivers may be forced to modify their signals, signaling behavior, and mate choice to reduce the unfavorable effects of eavesdropping (Bradbury & Vehrencamp, 2011; Hughes, Kelley, & Banks, 2012; Zuk & Kolluru, 1998). Long-distance acoustic signals are particularly

prone to exploitation by eavesdropping predators and parasites, and many studies have investigated their effects on prey signal design and signaling behavior most notably in birds, frogs, katydids, and crickets (Beckers & Wagner, 2012; Belwood & Morris, 1987; Cowles & Gibson, 2015; Lehmann & Heller, 1998; Tuttle & Ryan, 1982).

In frogs and toads, acoustic signals are the key modality for intraspecific communication (Gerhardt & Huber, 2002; Grafe, 2005; Toledo et al., 2015; Wells, 1977) and are directly linked to anuran diversification and evolution (Gerhardt, 1994). These calls are known to attract a variety of eavesdropping predators (Igaune,

Krams, Krama, & Bobkova, 2008; Jaeger, 1976; Smith, 1977; Tuttle & Ryan, 1981; Tuttle, Taft, & Ryan, 1981) and parasites (Bernal, Rand, & Ryan, 2006), a potentially major force in the evolution of anuran calling behavior and call design (Gomes, Halfwerk, Taylor, Ryan, & Page, 2017; Madelaire, José Da Silva, & Ribeiro Gomes, 2013; Pröhl, Eulenburg, Meuche, & Bolaños, 2013). Here, we explore the acoustic preferences of eavesdropping female frog-biting midges of the genus *Corethrella* that are attracted to the advertisement calls of male frogs, which act as their blood hosts (Camp & Irby, 2017; McKeever & Hartberg, 1977). We investigate the acoustic preferences of *Corethrella* spp. toward advertisement calls of syntopic frog hosts to determine the degree of host partitioning and how these preferences might influence the design of frog advertisement calls and frog calling behavior.

Although frog-biting midges have generated much interest for their unusual phonotactic behavior in recent years (Amaral & Pinho, 2015; Bernal et al., 2006; Borkent, 2008; ; Borkent & Grafe, 2012), many aspects of their interaction with anurans and the selective pressures they exert on their hosts remain poorly studied. Costs imposed by frog-biting midges on blood hosts could be substantial, ranging from irritation (indicated by defensive behaviors) and loss of blood (possibly substantial (Camp, 2006)) to an increased risk of infection with pathogens (Meuche, Keller, Ahmad Sah, Ahmad, & Grafe, 2016). Among such pathogens, trypanosome protozoans (Kinetoplastida: Trypanosomatidae) may represent the most important and diverse group of antagonists (Ferreira et al., 2015; Desser, 2001; Woo & Bogart, 1983), and acoustically oriented frog-biting midges are thought to be among the most relevant vectors of trypanosomes in frogs (Bernal & Pinto, 2016; Borkent, 2008; Johnson, Young, & Butler, 1993). Trypanosome infections can be pathogenic (Bardsley & Harmsen, 1973), and therefore, corethrellid preferences for host signal properties may create a strong selective force influencing signal evolution in their anuran host species. As yet there is no direct evidence for this, but Meuche et al. (2016) found that, among a set of Bornean frog species, those with midge-attractive calls were also the ones with the highest rates of trypanosome infections.

Although the ability to detect and exploit anuran advertisement calls likely has derived from a pre-existing sensory bias (use of acoustic signals during swarming (Silva, Nutter, & Bernal, 2015)), it seems likely that the corethrellid auditory system has adapted to maximize detection of frog hosts. However, the exact call parameters favored by selection are difficult to disentangle, and the extent to which those parameters differ between midge species and localities are essentially unknown (but see Grafe et al., 2018). In the present study, we aimed at identifying acoustic traits in frog calls that affect midge attraction around the La Gamba research station in Pacific lowland Costa Rica. We assumed that frog-biting midges have auditory preferences that will maximize host finding among the local anuran community. We measured the attractiveness of synthetic calls varying in dominant frequency and pulse duration as well as that of natural anuran advertisement calls recorded at the locality and broadcast at standardized sound pressure level with acoustic traps. We predicted that synthetic calls most closely resembling attractive natural calls

in call parameters (peak frequency and single call duration) would attract the largest number of midges.

2 | MATERIAL AND METHODS

2.1 | Study area

Experiments were performed between 2014 and 2018 at La Gamba (8°42'N, 83°12'W) in southern Costa Rica (www.lagamba.at). The research station is located near the Pacific coast at the edge of the Piedras Blancas National Park, one of Central America's last remaining areas of primary lowland tropical rainforest and one of the species-richest and most diverse forests in the world (Huber, Schaber, & Weissenhofer, 2017). Amphibian diversity at the study site is high, with at least 36 species of anurans being encountered in the vicinity of the station (Franzen & Kollarits, 2018). Acoustic trap experiments were performed at a large artificial swamp at the edge of the forest. The water level of the swamp was highly variable, ranging from completely dried out to >150 cm in depth. The species composition and abundance of calling frogs during/within the trials varied, depending on weather conditions and water level.

2.2 | Acoustic traps

Frog-biting midges can be caught in large numbers with acoustic traps broadcasting recorded anuran vocalizations (McKeever & Hartberg, 1980) or synthetic sounds (Bernal et al., 2006; Meuche et al., 2016). The common practice to capture frog-biting midges is the use of modified commercial insect traps that are equipped with a loudspeaker and have a battery-powered fan to suck in any midges attracted to the sound into a collecting chamber. Here, we used a new "bottle trap" method as a simple and (cost-) efficient alternative. The traps were made of 1.75 L modified plastic water bottles, with a speaker (Jay-Tech K10/A100) attached to the top end of the bottle (Figure 1). Female *Corethrella* spp. that approached the sound source emanating from within the bottle were drawn in and captured in water at the bottom of the bottle to which we had added a drop of odor-free detergent. For frequency response curves of the used speakers, see the digital appendix Figure S1 (measurement of speakers attached to traps, measured 1 m above trap entrance). All bottle trap tests were designed as choice experiments (= preference tests), with 2–10 traps being deployed simultaneously, placed in a row five meters apart from each other on ground level and displaying a different call or sound variant. Control traps, not broadcasting any sounds (i.e., silent), were included in each individual experiment, randomly placed in the trap lineup. Volume levels were adjusted using a Winpoon Digital Sound Level Meter at 1 m to a sound pressure level of 80 dB (dB re 20 µP; flat weighted and fast response setting), ensuring equal SPL between broadcast stimuli. The traps were run for 20–60 min from 18:00 to 24:00 hr, with up to four consecutive trials per night (no overlap of experiments). To evaluate the efficiency of our new trap design, we used a conventional fan-operated mosquito trap (BG Sentinel 2, Biogents AG) for a subset of the trials



FIGURE 1 Acoustic “bottle trap” used for this study. Acoustic stimuli were broadcast with a loudspeaker that was attached to the bottle. Frog-biting midges (*Corethrella* spp.) were captured in water (+ drop of detergent) at the bottom of the bottle, when approaching the sound source [Colour figure can be viewed at wileyonlinelibrary.com]

(advertisement calls of four focal species) to compare catch data. All captured midges were counted and transferred to EtOH p.a. for subsequent identification and further processing.

2.3 | Natural advertisement calls

Anuran advertisement calls were recorded at the study area with a Marantz PMD-561 portable digital recorder and a Rode NTG4 directional condenser microphone at a sample rate of 48 kHz and 24 bit resolution. For a comparative analysis of call parameters (see Figure 2), six calls per species (from six different specimens = calls used for acoustic trap experiments; presented below) were analyzed. Sound analysis was performed in Raven Pro (Vers. 64 1.5) using the following spectrogram parameters: Hann window, FFT window size 1,024 points, overlap 75%. Sound files for each target species were generated with Reaper (Vers. 5.311, Cockos Inc.) by extracting single calls of the recordings. To avoid pseudoreplication (see Kroodsma, Byers, Goodale, Johnson, & Liu, 2001), we used different calls (recorded from different specimens) from each target species for each individual trial. For each trial, sound files of 1 min were generated with 25 consecutive calls (one call variant), allowing for cross-comparability between species and artificial/natural call assays), with inter-call durations of 1 s (= segments of generated silence). The 1-min waveform was multiplied up to a total call display time of 30 min. The advertisement calls of the 10 most abundant anuran species (i.e., showing highest calling activity at the study site)

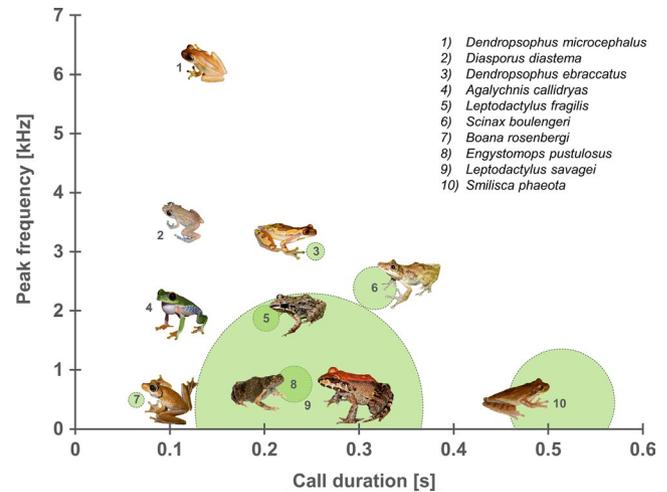


FIGURE 2 Advertisement calls of anuran species at La Gamba, Costa Rica, broadcast with acoustic traps; average peak frequency as a function of average single call duration. The bubble-size (diameter) represents median corethrellid trapping efficiency of the calls. Measurements were performed in Raven Pro (Vers. 64 1.5; 44.1 kHz, Hann window, FFT 1,024 points, 75% overlap). All sounds divided by a >50 ms—interval were defined as individual single calls, based on previous findings on pulse-discrimination (see Figure 6). In complex calls, consisting of multiple notes, the more frequently observed one was used for visualization (e.g., only the “whine” for *Engystomops pustulosus*) [Colour figure can be viewed at wileyonlinelibrary.com]

from three frog families were used for the experiments with acoustic traps: Hylidae: *Agalychnis callidryas*, *Boana rosenbergi*, *Dendropsophus ebraccatus*, *Dendropsophus microcephalus*, *Scinax boulengeri*, *Smilisca phaeota*; Eleutherodactylidae: *Diasporus diastema*; Leptodactylidae: *Engystomops pustulosus*, *Leptodactylus fragilis*, *Leptodactylus savagei*. Bufonid species also regularly encountered calling at the site (*Incilius coniferus*, *Rhinella marina*) were not included in this test approach, due to their extensive trilling advertisement calls, hindering comparability with short “single-impulse” calls. For each trial, six traps (i.e., five different species’ calls + one control) were deployed simultaneously, each 5 m apart, with call combinations being permuted randomly. A total of six trials were carried out over a period of one week (June 2018). Further, we tested whether the temporal call structure influenced trapping efficiency, based on distinct recognition of complex temporal patterns of modulations (frequency and amplitude) within a specific call. For this, natural calls of three target species (*E. pustulosus*, *L. savagei*, *S. phaeota*) were tested against identical calls that were played backward (reverse-playback) in a pairwise comparison (4–10 trials). Comprehensive call data and the sound files used for the experiments can be made accessible upon request.

2.4 | Synthetic calls

To independently evaluate the influence of spectral (peak frequency) and temporal call patterns on positive phonotaxis in frog-biting midges, artificial calls (sinusoidal pure tones) were broadcast with

acoustic traps and tested for attractiveness in simultaneous choice tests (preference tests). These synthetic calls (subsequently referred to as pulses) were generated in Audacity (Vers. 2.0.5) at different frequencies and with different pulse durations. For any given trial, a max. total of 10 traps was deployed simultaneously for 60 min and each trap was randomly assigned to one of the test pulses. The following experiments were conducted:

2.4.1 | Frequency dependence

To evaluate frequency preferences of frog-biting midges during phonotactic foraging, pulses with different frequencies were broadcast, covering a range from 200 Hz to 8.2 kHz (150 Hz-steps from 200 to 3,200 Hz; 1 kHz-steps from 3,200 to 8,200 Hz). Pulses were generated using a constant pulse duration and inter-pulse duration (generated silence) of 1 s each. Each frequency was tested 3–8 times within a four-week period (March–April 2014), in randomized groupings (with replacement) of 10 traps/pulse variants per trial (+ control trap).

2.4.2 | Pulse duration dependence

Constant duty cycle

Temporal preferences of frog-biting midges were tested using 350 Hz pure-tone pulses of different durations. The test frequency was chosen based on its effectiveness in attracting *Corethrella* in preliminary trials. Pulses were separated by equally long inter-pulse intervals to generate sound files with equal duty cycles. Pulse durations ranged from 125 ms to a continuous tone in the following steps ([s]: 0.125–0.25–0.5–1–2–4–8–16–32–continuous). Each pulse duration was tested 4–7 times within a four-week period (March–April 2014).

Constant inter-pulse duration

For this experiment, we also used sinusoidal pure sounds at 350 Hz, but used a different range of pulse durations ([s]: 0.062–0.125–0.25–0.5–1). In contrast, the inter-pulse duration was fixed to 1 s, resulting in equal inter-pulse durations but variable duty cycles that increased with pulse duration. To ensure that differences in trapping rates were not based on different total numbers of pulses broadcast, the number of successive pulses was fixed to 25/min. Trials were repeated 15 times over a period of four months (June–September 2015).

2.4.3 | Inter-pulse duration dependence

In preliminary tests, we found that frog-biting midges were not being attracted to continuous sounds (pure sine waves) broadcast over extended time periods (several min). To determine the minimum inter-pulse duration that frog-biting midges need to locate sounds, the inter-pulse duration was varied from 5 to 100 ms with a constant pulse duration of 1 s and limited repetition of 25 consecutive pulses per minute. A continuous tone of 25 s duration (per minute) was broadcast as a control. Trials were repeated 15 times over a period of four weeks (April 2016).

2.5 | Statistical analyses

We used generalized linear mixed modeling (GLMM) to analyse *Corethrella* catches in acoustic traps. Analyses were performed in R Studio (V. 1.0.143) using the lme4 and emmeans packages (Bates, Mächler, Bolker, & Walker, 2014; Lenth, 2019). We specified the respective acoustic stimuli (i.e., natural frog calls, different synthetic call models) as fixed effects to test for variability in midge attraction. As all tests were designed as choice experiments (i.e., multiple acoustic stimuli with overlap of active space), sounds eliciting either a positive or negative phonotactic response could potentially affect catch numbers in other traps of the same trial. We addressed these difficulties by randomizing stimulus combinations within trials and a high number of trials (repetitions). For statistical analyses, we thus treated each trap as independent, based on the following reasoning: (a) catch proportions and species composition in choice experiments did not differ from those of single-stimulus tests (see results: comparison of trap designs); (b) sounds from acoustic traps blended in with the naturally variable (anuran) acoustic background at the study site, with abundance and composition of calling hosts varying due to specific activity cycles and/or changes in weather conditions. To further control for such context effects in our models, we included “day” and (if applicable) different “trapping time window” (6-7/7-8/8-9/9-10 p.m.; nested within day) as random effects (intercepts). We performed likelihood ratio estimations (Laplace approximation) for each experimental approach to test for deviation from the null hypothesis (no differences in catch numbers). Reference parameters for each model were set to the predictor variable showing the highest catch numbers. As our residuals indicated overdispersion when data were treated as Poisson (log-link), we re-fitted our models using a negative binomial distribution for the response variable (i.e., count data of midges). In case of significant main effects, we performed pairwise comparisons (Tukey HSD) to assess contrasts in catch numbers among selected frequencies, pulse, and inter-pulse durations. To compare abundance distributions of midge species attracted to traps (i.e., different acoustic stimuli), we performed Fisher's exact tests implemented in the *aylmer* package (West & Hankin, 2008). To test the effects of peak frequency and single call duration on median captures among natural advertisement calls, we used Spearman's rank correlation in Statistica (V. 13).

2.6 | Morphological species identification

Midges of each sample (= each individual trap, all natural advertisement calls, all test frequencies, and pulse durations) were morphologically examined with a total maximum of 100 midges per sample (sample sizes <100 were assessed completely). To avoid observer bias, all midge subsamples were picked blindly from the main samples (EtOH). Midges were categorized based on morphological features using the characters in the key to new world species of Corethrellidae (Borkent, 2008). Representative individuals were mounted on microscopic slides using Entellan[®] rapid mounting

medium (Merck Millipore) and identified to species by A. Borkent, Salmon Arm, British Columbia, Canada.

2.7 | Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All field experiments and collections were conducted under permissions granted by the Costa Rican National System of Conservation Areas (SINAC) and the National Commission for the Management of Biodiversity (Conagebio) (permission IDs: INV-ACOSA-036-2015; R-007-2016-OT-CONAGEBIO; SINAC-ACOSA-PI-PC-078-18).

3 | RESULTS

Overall, the data showed great variability in attraction (total catch numbers and species proportions) of frog-biting midges to both natural calls and artificial call models. No midges were attracted to silent control traps, indicating that neither bottles nor the trapping liquid (water and detergent) emitted chemical or visual midge-attractive stimuli. We identified six morphologically different midge species in our traps, four of which are known species, and two of which were not yet identified species of *Corethrella* (see below). The relative abundance of the three most common *Corethrella* spp. in acoustic traps ($N = 21,489$ morphologically examined specimens) remained largely unchanged between trapping years 2014–2018 (Fisher's exact test, $p = 0.65$), allowing for a cross-comparability of the different test approaches over the different years. Although total catch numbers of *Corethrella* varied between days and trapping time, both factors accounted for only a small part of the variation in our GLMMs (see digital appendix, Table S1a). Only for two models (synthetic calls: frequency and pulse duration dependence), the integration of trapping day as a random factor resulted in a significantly better model fit, based on AIC/BIC and X^2 estimations (see digital appendix, Table S1b).

3.1 | Comparison of trap designs

The bottle trap method proved to be effective for capturing frog-biting midges in high numbers during our trials. However, compared to fan-operated mosquito traps, the trapping efficiency per unit time was lower: 30-min trap assays with bottle traps brought in similar numbers of midges as 5-min assays with the fan-operated alternative (see digital appendix Figure S2). Importantly, abundance distributions of midge species did not differ between the different trap types (preference tests with bottle traps vs. approach tests with BG mosquito traps; comparison of overall catch numbers, Fisher's exact test, $p = 0.68$). Water and detergent in the bottle traps did not compromise species identification (e.g., due to loss of scales, bleaching) in most cases. Only when specimens were kept in the water for longer duration (overnight), species identification became more difficult.

3.2 | Natural advertisement calls

The number of trapped midges (total catch numbers, all species) varied significantly among calls of different frog species, with considerable variation between the tested calls (GLMM, $n = 60$, $z = 21.03$, $p < 0.0001$; for details of GLMM-results, see digital appendix Table S2a). Calls of the Giant Bullfrog (*L. savagei*) attracted by far the largest number of midges (median = 732 midges per 30 min), with maximum values reaching up to 2,960 midges in 30 min. This call attracted up to 14 times more midges than the calls of other attractive species (for median numbers of midges per hour, see digital appendix Figure S3).

Trapping efficiency correlated negatively with frequency (Spearman's rank correlation with rounded medians; $R_s = -0.79$, $N = 10$, $p = 0.0065$) and positively with call duration ($R_s = 0.77$, $N = 10$, $p = 0.0098$). Figure 2 shows catch numbers of traps displaying the recorded calls, plotted as a function of call peak frequency and single call duration. The advertisement calls showing the highest trapping efficiency in our experiments (*L. savagei* [9], *S. phaeota* [10]) were the calls with the lowest peak frequencies (373/462 Hz). The remaining species with somewhat attractive calls were all found either in a range of low peak frequencies (<1 kHz) or/and call durations between ~200 and 300 ms. Spectrogram analyses also showed, that the calls of *L. fragilis* [5], *S. boulengeri* [6] and *D. ebraccatus* [3] were broadband with energy below their peak frequencies, with fundamental frequencies ranging from 0.95 to 2 kHz, likely affecting their attractiveness.

To test whether the observed differences in the attractiveness of the calls of different species were based on differences in the temporal structure of the call, for example, distinct temporal modulations of frequency and amplitude, three of the most attractive calls (*L. savagei*, *S. phaeota*, *E. pustulosus*) were tested pairwise in natural versus reversed call playback (see digital appendix Figure S4). In no case were there significant differences in the numbers of attracted midges (Tukey HSD, $p = 0.28$ – 0.92 ; for a summary of all pairwise Tukey HSD, see digital appendix Table S2b).

The abundance distribution of midge species in samples of traps broadcasting natural frog advertisement calls was also highly skewed. Out of 3,450 morphologically examined midges, *C. ranapungens* was the most abundant species by far, with 93.4% of all samples. *C. amazonica*/*C. ramentum* (subsequently treated together, as these species were only clearly distinguishable when mounted on microscopic slides) represented 5.7% of catches, and *C. peruviana* represented 0.6%. The remaining specimens consisted of the rare *C. cf. quadrivittata*, (0.1%) and two additional species that could not be identified so far 0.2%.

For the seven most attractive calls (i.e., those with numbers allowing meaningful comparison: *D. ebraccatus*, *E. pustulosus*, *B. rosenbergi*, *L. fragilis*, *L. savagei*, *S. boulengeri*, *S. phaeota*), the composition of the three most common species was significantly different for most pairwise comparisons (Fisher's exact tests, for p values see digital appendix Table S3). Calls of all frog species attracted large numbers of *C. ranapungens*, which particularly

dominated the catches of traps displaying *L. savagei* calls (Table 1). In contrast, the calls of *B. rosenbergi* also attracted substantial numbers of *C. peruviana* (6% of all catches), with multiple specimens being attracted in each trial (additionally it was only found as singletons on two occasions in traps broadcasting the call of *L. fragilis*). Specimens of *C. amazonica/C. ramentum* were found in variable proportions in all traps of the more efficient (i.e., consistently showing high catch numbers) frog calls, with the highest numbers also being found in traps broadcasting the calls of *B. rosenbergi*. We found that catch proportions in trials varied significantly between trapping time windows (1–4) for the three most common midge species (Fisher's exact test, $p < 0.001$). However, rarer species were not reduced in later trials (compare digital appendix Table S4), indicating that multiple consecutive trials per night did not generally bias against rarer midge species. Observed differences in catch proportions between trials are likely to be influenced by variations in calling activity and composition of frog species at the site. However, at this point, we only have limited information on midge activity cycles and the relevant (environmental) impact factors contributing to observed small-scaled temporal and spatial variation in abundance.

3.3 | Effects of frequency

Catch numbers differed greatly among traps displaying artificial sounds of variable frequencies (Figure 3). Frequencies of 200–650 Hz showed the highest catch numbers, with a maximum at 500 Hz (median of 449 trapped midges/hr). At higher frequencies, capture rates dropped abruptly to below 9 midges/h. Above 1550 Hz, only single midges were trapped occasionally. No midges were caught in traps broadcasting sounds above 4.2 kHz (not displayed in Figure 3). Within the statistically tested range of 200–1550 Hz, the attractiveness varied significantly among test frequencies (GLMM, $n = 61$, $z = 10.88$, $p < 0.0001$). Pairwise comparisons (Tukey HSD) between the most attractive frequency, 500 Hz, and the remaining frequencies showed significant differences ($p < 0.05$) in the number of midges trapped for all frequencies, except 200 Hz ($p = 0.24$) and

350 Hz ($p = 0.97$). Differences between 200/650 Hz were also not significant ($p = 0.52$). Thus, the spectral bandwidth preferred by *Corethrella* in La Gamba ranged from 200 to 650 Hz.

Acoustic traps broadcasting sinusoidal tones at different frequencies were heavily dominated by *C. ranapungens*: 97.3% of all examined individuals (total $n = 1723$) were *C. ranapungens*, while only 2.5% were *C. amazonica/C. ramentum*. The distribution of the two species across traps displaying different frequencies was significantly different (Fisher's exact test: $p < 0.0001$). Both were maximally attracted to 500 Hz, but the preference of *C. amazonica/C. ramentum* for that frequency was more pronounced (74.4% of individuals), indicating that the spectrum of preferred frequencies is broader for *C. ranapungens*.

3.4 | Effects of pulse duration

In experiments with constant duty cycle, that is, variable inter-pulse duration, midges were attracted only at pulse durations ≤ 16 s, with median numbers of catches decreasing with increased pulse duration (Figure 4), whereas continuous tones over the complete 60-min period and 32-s pulses did not attract any midges. Within the statistically tested range < 16 s, the number of attracted midges differed significantly between traps (GLMM, $n = 52$, $z = 14.60$, $p < 0.0001$), with maximum attraction at 0.125 s (median $n = 430$).

In experiments with constant inter-pulse durations, that is, variable duty cycle (Figure 5), there was a significant difference in the number of midges trapped (GLMM, $n = 67$, $z = 36.13$, $p < 0.0001$), with highest attraction between 125 and 500 ms (maximum at 250 ms: median $n = 174$ /hr). Midge captures decreased toward shorter and longer pulses, showing significantly lower catch numbers for most pairwise comparisons, except for 125/500 ms and 250/500 ms (Tukey HSD: $p = 0.80$ and $p = 0.23$, respectively). An increase in successive pulse numbers led to a proportional increase in midges attracted, whereas the overall distribution of catches (i.e., pulse preferences) remained similar (data not presented).

In traps broadcasting sounds with different pulse durations (at 350 Hz) two *Corethrella* species were found with the following

TABLE 1 Total numbers and proportions (%) of morphologically identified *Corethrella* spp. found in acoustic traps broadcasting anuran advertisement calls

	<i>C. ranapungens</i>	<i>C. amazonica/C. ramentum</i>	<i>C. peruviana</i>	<i>C. cf. quadrivittata</i>	Unidentified
<i>A. callidryas</i>	79 (97.6)	1 (1.2)	0 (0.0)	0 (0.0)	1 (1.2)
<i>D. diastema</i>	88 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>D. ebraccatus</i>	374 (95.2)	19 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)
<i>D. microcephalus</i>	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>E. pustulosus</i>	475 (92.8)	32 (6.2)	0 (0.0)	2 (0.4)	3 (0.6)
<i>H. rosenbergi</i>	205 (63.9)	96 (29.9)	19 (5.9)	1 (0.3)	0 (0.0)
<i>L. fragilis</i>	401 (98.0)	4 (1.0)	2 (0.5)	0 (0.0)	2 (0.5)
<i>L. savagei</i>	498 (99.6)	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)
<i>S. boulengeri</i>	532 (98.0)	9 (1.6)	0 (0.0)	0 (0.0)	2 (0.4)
<i>S. phaeota</i>	566 (94.4)	32 (5.3)	0 (0.0)	2 (0.3)	0 (0.0)

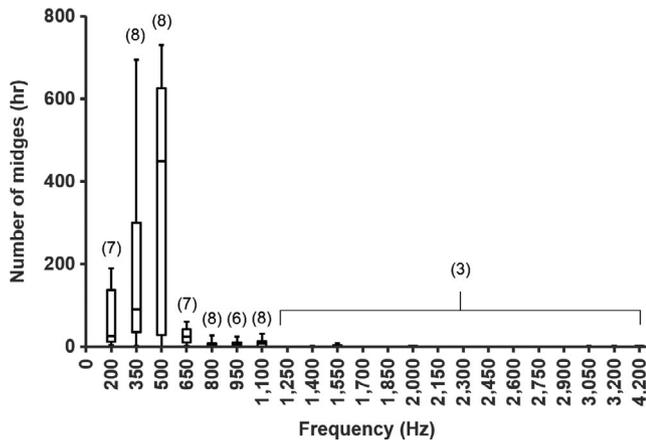


FIGURE 3 Numbers of midges (*Corethrella* spp.) caught with acoustic traps broadcasting sinusoidal pure tones of different frequency in La Gamba, Costa Rica (N = 3–8 per frequency; see brackets). Box-Whisker plots indicate numbers of midges per 1h-trial (median, Q0.25, 0.75, min–max). Pulses of 1 s duration were alternated with inter-pulse durations of 1 s

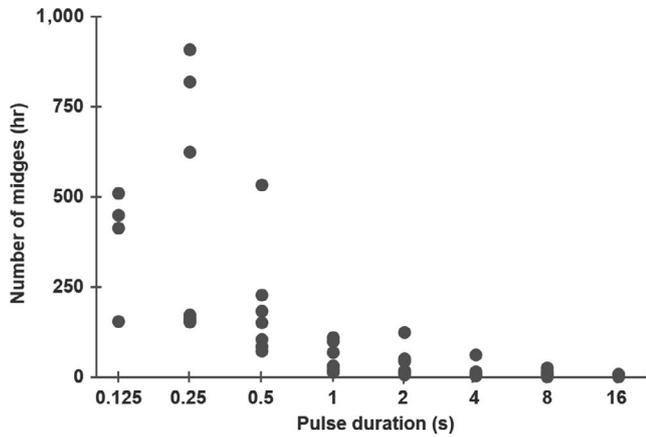


FIGURE 4 Relationship between pulse duration and number of midges (*Corethrella* spp.) caught with acoustic traps (runtime: 1 hr) in La Gamba, Costa Rica. The scatterplot shows absolute trapping numbers per trial (N = 4–7 per pulse duration; note logarithmic scale on x-axis). Sinusoidal pulses were presented at 350 Hz. The pulse duration equaled the inter-pulse duration

proportions (data corresponding to Figure 4; total number of specimens: 2,476): *C. ranapungens* 98.3%, *C. amazonica/C. ramentum* 1.7%. The abundance distribution of midge species attracted to different pulse durations varied significantly between the two species (Fisher's exact test, $p < 0.001$). *C. ranapungens* was found in high proportions at all test pulses, whereas *C. amazonica/C. ramentum* showed the highest attraction to pulses with a 1 s–duration. However, overall catch numbers were low for this species, so these findings have to be interpreted carefully.

3.5 | Effects of inter-pulse duration

When we varied the inter-pulse duration, we found that very short interruptions of only 5 ms were sufficient to trigger positive

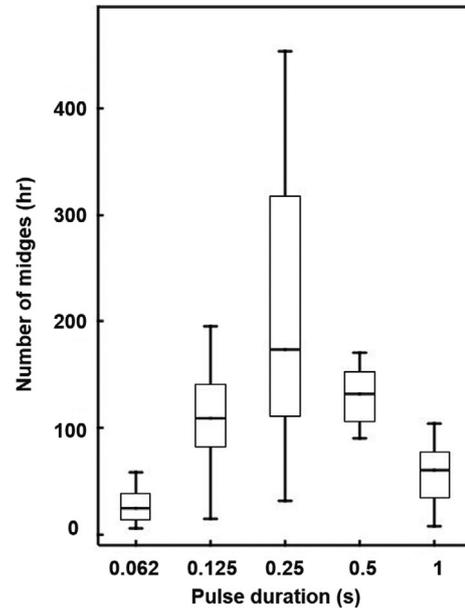


FIGURE 5 Number of midges (*Corethrella* spp.) caught with acoustic traps broadcasting synthetic sounds varying in pulse duration in La Gamba, Costa Rica. Box-Whisker plots show median number of midges per 1h-trial (Median, Q0.25, 0.75, min–max). Sinusoidal pure tones (350 Hz) were generated with variable pulse durations (0,062 s–1 s), with constant inter-pulse duration of 1 s (N = 15), with a pulse rate of 25 consecutive pulses/min. See Table S2 for pairwise statistics

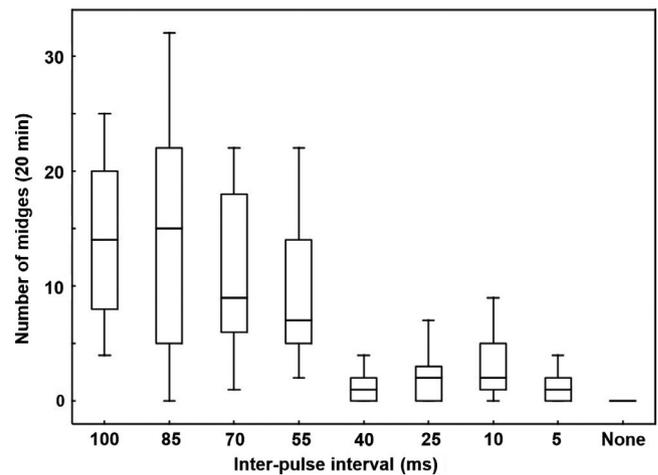


FIGURE 6 Number of midges (*Corethrella* spp.) caught with acoustic traps broadcasting synthetic sounds varying in inter-pulse duration in La Gamba, Costa Rica. Box-Whisker plots show median number of midges per 20-min trial (Median, Q0.25, 0.75, min–max). Sinusoidal tones (pulse duration of 1 s; 25 consecutive pulses/min; generating frequency 350 Hz) with variable inter-pulse duration (5–100 ms) were tested versus a continuous 25 s–tone (N = 15)

phonotaxis in *Corethrella* spp., whereas a continuous tone (25 s) did not attract any midges at all (Figure 6). However, catch numbers were low at inter-pulse durations of 5–40 ms (median $n = 1$ –2). At 55 ms interval, we found a significant increase in the number of midges trapped (median $n = 7$) compared to all shorter interval durations

(Tukey HSD, all $p < 0.01$). Trapping efficiency appeared to increase slightly with interval prolongation, but differences within the range of 55–100 ms were not significant (Tukey HSD, $p = 0.53$ – 0.99).

4 | DISCUSSION

This study comprehensively explored auditory preferences of frog-biting midges in lowland Pacific Costa Rica, using both natural and artificial calls and covering an extensive range of frequencies and pulse durations in high resolution. We found that midge attraction was influenced by both spectral and temporal call parameters. Low frequencies (<1 kHz) and short pulse durations (250–500 ms) attracted the largest numbers of midges in tests with synthetic sinusoidal pure tones. The attractiveness of natural frog call playbacks was explained in part by those findings, but some of the variation remained unexplained (see below). Our bottle trap design proved to be effective in catching high numbers of midges and thus can be recommended as a cost-efficient alternative to well-established mosquito traps. However, the comparatively low trapping efficiency per time unit may limit its usability in some cases and general advantages/disadvantages of multiple-stimulus approaches have to be considered independently for each test design.

4.1 | Acoustic preferences

The preferred frequency bandwidth found in the midges from La Gamba was 200–650 Hz, with an effective upper threshold at ~4 kHz. Similar upper limits to hearing have been found in frog-biting midges from southern Brazil (Caldart, Santos, Iop, Pinho, & Cechin, 2016) and Borneo (Meuche et al., 2016). Frog-biting midges were not attracted to continuous tones (>16 s) but clearly required pulsed auditory stimulation (on–off patterns) with very short inter-pulse durations of down to 5 ms being sufficient for stimulus recognition. This suggests that frog-biting midges detect and locate host calls using onset and/or offset acoustic cues known to play an important role in insect communication (Balakrishnan, Von, & Von, 2001). Further, this also enables frog-biting midges to detect complex calls consisting of multiple short notes or trilling (“pulsed”) advertisement calls (e.g., found in many bufonid species) that blend in with the ambient noise.

The attractiveness of recorded natural calls varied greatly, as it has been shown in previous studies (Borkent, 2008; Caldart et al., 2016; McKeever & French, 1991; Meuche et al., 2016), suggesting a selective perception or preference for certain call characteristics. The advertisement call of the Giant Bullfrog (*L. savagei*) was most attractive to frog-biting midges, more than an order of magnitude more attractive than the calls of other frogs. Notably, all calls that were efficient (i.e., consistently showing high catch numbers) in attracting midges were found in either the most efficient range of peak frequencies (200–650 Hz) or pulse durations (250–500 ms) as revealed by artificial call models, with the most efficient calls (*L. savagei*, *S. phaeota*) matching both criteria. This indicates that both spectral (peak frequency) and temporal (single call duration) are important

parameters in host finding, corroborating the findings of previous studies (Aihara, Silva, Bernal, & Wright, 2016; Bernal, Page, Rand, & Ryan, 2007; Meuche et al., 2016). However, it is likely that additional spectral parameters (e.g., fundamental frequency, harmonics) take effect during the midges' phonotaxis, indicated by the effectiveness of three of our test calls (*D. ebraccatus*, *L. fragilis*, *S. boulengeri*) that could not be explained by their peak frequencies but were presumably based on lower fundamental frequencies. We further found, that within the range of tested call manipulations (reverse calls), there was no evidence for call recognition and preference, based on a distinct temporal structure of individual pulses, making overlap in attraction to different calls with similar parameters likely. However, it is possible that certain call modulations (e.g., amplitude- or frequency-modulations) contrasting with ambient noise are generally used during phonotaxis as well. It seems likely that contextual and environmental parameters (e.g., perch height and vegetation density affecting the degree of attenuation and scattering (Morton, 1975; Gerhardt & Huber, 2002), ambient noise masking frog calls and preventing detection (Bee, 2012)) influence the attraction rates (i.e., call recognition and localization performance) of foraging frog-biting midges and thus should be integrated in future investigations.

4.2 | Midge diversity and host specificity

A total of six morphologically different species were caught in acoustic traps in La Gamba, Costa Rica: *C. ranapungens*, *C. amazonica*/*C. ramentum*, *C. peruviana*, *C. cf. quadrivittata*, and two yet unidentified species only found as singletons. Preliminary molecular genetic data (JV unpublished) suggest that at least one of these species (*C. ranapungens*) harbors additional cryptic species, so the true midge diversity in acoustic traps is likely to be higher. Additional corethrellid species have also been found in La Gamba by directly sampling midges from calling frogs (Virgo et al. in prep.).

The quantitative distributions of the two midge species found in traps displaying artificial call models indicate that spectral and temporal call preferences vary among *Corethrella* spp. Although both were attracted to a broad range of test frequencies, *C. amazonica*/*C. ramentum* showed a more pronounced preference for traps broadcasting pulses at 500 Hz. At the same time, the preferred pulse durations (at 350 Hz) varied between the two species. However, these findings have to be verified by additional experiments, applying different pulse durations to a broader range of test frequencies.

All *Corethrella* spp. were found in traps displaying calls of a range of different frog species. This suggests that there is no general close species-level host specificity (based on distinct acoustic cues) in corethrellids in La Gamba. Similar observations from other investigations using sound traps also suggest that frog-biting midges use a more generalized acoustic template, allowing for a wide host spectrum and the ability of seasonal host switching (Legett, Baranov, & Bernal, 2018). However, for the three most common midge species, that is, those with numbers allowing meaningful comparison, the quantitative distribution of individuals across traps/frog calls was significantly different, suggesting some level of acoustic niche

differentiation. *C. ranapungens*, the most common species overall, showed broad attraction to most acoustic stimuli presented. It had a strong preference for the call of the leptodactylid species *L. savagei*, whereas *C. amazonica/C. ramentum* and *C. peruviana* were more strongly attracted to calls of the hylid species *B. rosenbergi*. The preference of *C. amazonica/C. ramentum* for *B. rosenbergi* calls is in agreement with its preferences for artificial call models with a frequency of 500 Hz, which matches the peak frequency of this species (502 Hz). High catch rates of this species were also observed for advertisement calls of *S. phaeota* and *E. pustulosus*, as well roughly matching the preferred spectral properties (peak frequencies of 463 and 768 Hz, respectively). Although absolute numbers were comparatively low, *C. peruviana* appeared overrepresented in traps broadcasting calls of *B. rosenbergi*, indicating that its call matches the acoustic preferences of this midge species. Further examination will be necessary to verify these observations and to assess the relevance of *B. rosenbergi* as a potential host for *C. peruviana*.

It should be emphasized that a comparison purely based on sound-trap catches probably underestimates the true levels of host specificity, potentially excluding other non-auditory mechanisms of host recognition, for example, chemical or mechanical cues that may be only effective upon landing. Further, our experiments did not account for potential specialization in midges based on foraging heights. Thus, the placement of traps on ground level might also introduce certain bias, as some of the tested frog species are typically found calling from elevated perches. Indeed, our preliminary analyses of midges collected directly from frogs show more pronounced host preferences, with some species showing a narrower host range than others (Virgo et al. in prep.). In a recent study using ecological network analyses, Grafe et al. (2018) found both specialized and generalized midges when sampling on frogs in Borneo, suggesting that some frog species are better at avoiding being bitten by *Corethrella* than others.

It should also be noted that the midge catches at our site were highly dominated by one species, *C. ranapungens*, which represented 96% of all catches in acoustic traps. Similarly, high abundance proportions for this species were found at Gamboa, Panama (Leggett et al., 2018), indicating that it is at least locally highly abundant and potentially widespread throughout southern Central America. However, preliminary molecular data indicate that this species harbors multiple genetically distinct lineages (Virgo et al. in prep.), thus levels of specificity might be underestimated at this moment. More comprehensive data on distribution patterns and thorough molecular genetic investigations will be needed to assess these assumptions. We found a close correspondence in the relative proportions of midges captured with acoustic traps (both trap types) and those collected directly from frog hosts (JV unpublished). Similar observations were made in peat swamps of Brunei (Grafe et al., 2018), suggesting that acoustic traps accurately reflect true midge abundances. Other methods, for example, resting boxes (Camp, 2006), could additionally be included in future studies to obtain more accurate estimations of midge abundances as well as species diversity.

4.3 | Are there key blood hosts?

As yet, there is only limited information on what exactly constitutes a suitable blood host for frog-biting midges and the relevant cues eliciting host choice. Certain call parameters might be indicative of host qualities, like body size (inverse correlation between body size and dominant frequency (Gingras, Boeckle, Herbst, & Fitch, 2013)) or host density (e.g., male túngara frogs increase call complexity in presence of additional vocalizing males (Rand & Ryan, 1981; Bernal et al., 2007)), and it is possible that frog-biting midges use such traits to maximize foraging efficiency (Bernal et al., 2007). Large frogs might be preferable blood hosts as observed levels of defensive reactions to *Corethrella* attacks appear to be lower than in smaller frog species (JV pers. obs.). The preference for low-frequency calls might thus be the result of a generalist sensory tuning to larger species' calls. Such "universal" preferences for acoustic traits have been described for female frogs (Ryan & Keddy-Hector, 1992) and might apply for *Corethrella* as well. However, frequency preferences/acoustic templates appear to differ between localities (compare Meuche et al., 2016), indicating that sensory tuning in *Corethrella* might have evolved in close correspondence to local host communities. The outstanding attractiveness of the Giant Bullfrog's call (*L. savagei*) to midges agrees with direct observations made at the study site: On several occasions, large numbers (up to >50) of aggregating midges could be collected from this host. Calling males were observed to continue calling while being literally covered with hundreds of corethrellids, and males showed no attempts to repel the midges (Figure 7). This frog (as part of the *L. pentadactylus*-species group; Heyer, 2005) is abundant and widespread throughout Central America and shows an extended calling activity throughout the wet season. Its large body size and an apparent lack of defensive behaviors may contribute to *L. savagei* being a particularly suitable blood host, which might have disproportionately influenced the evolution of corethrellid auditory tuning and host-locating behavior. Comparative studies at a range of localities in Central America are necessary to corroborate this hypothesis.



FIGURE 7 Frog-biting midges (*Corethrella* spp.) attacking a male Bullfrog (*Leptodactylus savagei*). The male started calling in response to a nearby sound trap deployed during the dry season. (Photo: T. Eltz, La Gamba 03/2019) [Colour figure can be viewed at wileyonlinelibrary.com]

Conclusively, our data indicate that frog-biting midges use rather generalist acoustic templates to detect suitable blood hosts. Being based on elementary spectral and temporal call parameters, these templates only allow for low levels of acoustic niche differentiation, whereas higher levels of host specificity are likely to be based on a multimodal perception of additional non-acoustic (e.g., chemical) cues, in the close range. It is likely that acoustic preferences (i.e., acoustic templates) in *Corethrella* have evolved in close correspondence to local host communities; however, we still know very little about the exact parameters favoring selection for certain host species and/or call variants. To address these questions, it is to be investigated in how far acoustic preferences of *Corethrella* spp. differ on a regional and transregional scale and in how far potential differences can be associated with local host communities.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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