



Acoustic Preference of Frog-Biting Midges (*Corethrella* spp) Attacking Túngara Frogs in their Natural Habitat

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Received: July 18, 2015

Initial acceptance: September 28, 2015

Final acceptance: November 1, 2015

(J. Wright)

doi: 10.1111/eth.12452

Keywords: acoustic preferences, eavesdroppers, call rate, call complexity, *Physalemus*

Abstract

In many animals, males aggregate to produce mating signals that attract conspecific females. These leks, however, also attract eavesdropping predators and parasites lured by the mating signal. This study investigates the acoustic preferences of eavesdroppers attracted to natural choruses in a Neotropical frog, the túngara frog (*Engystomops pustulosus*). In particular, we examined the responses of frog-biting midges to natural variation in call properties and signaling rates of males in the chorus. These midges use the mating calls of the frogs to localize them and obtain a blood meal. Although it is known that the midges prefer complex over simple túngara frog calls, it is unclear how these eavesdroppers respond to natural call variation when confronted with multiple males in a chorus. We investigated the acoustic preference of the midges using calling frogs in their natural environment and thus accounted for natural variation in their call properties. We performed field recordings using a sound imaging system to quantify the temporal call properties of males in small choruses. During these recordings, we also collected frog-biting midges attacking calling males. Our results revealed that, in a given chorus, male frogs calling at higher rates and with higher call complexity attracted a larger number of frog-biting midges. Call rate was particularly important at increasing the number of midges attracted when males produced calls of lower complexity. Similarly, call complexity increased attractiveness to the midges especially when males produced calls at a low repetition rate. Given that female túngara frogs prefer calls produced at higher repetition rates and higher complexity, this study highlights the challenge faced by signalers when increasing attractiveness of the signal to their intended receivers.

Introduction

In lekking species, males aggregate to engage in competitive displays to attract females. In species of anurans and insects that form leks, most often in the form of choruses, males in such aggregations enjoy higher mating probabilities in larger choruses than in smaller ones (Ryan et al. 1981; Cade & Cade 1992; Wagner & Reiser 2000; Gray & Eckhardt 2001). By displaying in a group, males also enjoy lower per capita predation risk (Ryan et al. 1981). Although the relative role of these benefits in the evolution of chorusing behavior

is unclear (Gerhardt & Huber 2002), competition between males is intensified by displaying in aggregations. Displaying in groups results in selection for traits that increase signal attractiveness to females compared to the signals produced by neighboring males. Signal traits that deviate from the mean value in the population are usually preferred by conspecific females (Ryan & Keddy-Hector 1992). Playback experiments demonstrate that female gray tree frogs (*Hyla versicolor*), for instance, prefer longer calls of conspecific males than shorter calls (Gerhardt et al. 2000). Similarly, female African painted reed frogs

(*Hyperolius marmoratus*) prefer lower frequency calls than higher frequency calls (Jennions et al. 1995). Individual strategies to maximize female attraction are common within the chorus. These attempts to increase attractiveness to conspecific females, however, may also increase conspicuousness of the signal to unintended receivers and elevate the attack rates by eavesdropping predators and parasites (Zuk & Koluru 1998). Signaling strategies relative to competing males are thus at the core of the conflict between sexual and natural selection.

In this study, we investigate the attacks of eavesdropping parasites to calling males in a Neotropical anuran species in which the signal preferences of females have been widely examined. Túngara frogs (*Engystomops*, *Physalaemus*, *pustulosus*) are a common anuran species found in Middle and Northern South America. Male túngara frogs aggregate to vocalize in puddles and ditches to attract conspecific females (Ryan 1985). Their advertisement calls consist of frequency-modulated sweeps, called 'whines' (Fig. 1). Shorter, secondary complex components called 'chucks' can be facultatively added by males at the end of the whine (Fig. 1; Rand & Ryan 1981; Ryan 1985). Males can add up to seven chucks to a single whine when competing with other calling males (Bernal et al. 2007). These added components increased the attractiveness of the calls to female túngara frogs (Rand & Ryan 1981).

While the advertisement calls of túngara frogs are highly effective at attracting conspecific females, they are also attractive to unintended receivers that exploit this communication system. Various species of predator and parasite eavesdroppers cue in túngara frog

advertisement calls including frog-eating bats (*Trachops cirrhosus*), frog-biting midges (*Corethrella* spp), South American bullfrogs (*Leptodactylus pentadactylus*), and four-eyed opossums (*Philander opossum*) among others (reviewed in Page et al. 2014). In this study, we focus on the most abundant eavesdropper in this system, the frog-biting midges (Fig. 2a; Bernal et al. 2006). Although it has long been known that the advertisement call produced by túngara frogs is sufficient to attract frog-biting midges (Bernal et al. 2006), recent work has shown that the call is the only long-distance cue necessary for these eavesdroppers to detect and locate their host and ultimately obtain a blood meal (Bernal & de Silva 2015). In addition to taking a blood from calling male frogs, these midges act as vectors of a blood parasite, *Trypanosoma tungarae* (Bernal & Pinto, in review). Two-speaker playback experiments with a fixed call rate revealed that frog-biting midges prefer calls with chucks to simple whines (Bernal et al. 2006). The role of other call features at affecting frog-biting midge attacks, however, is unknown. Although call complexity may be relevant when other call features are held constant, natural variation in call characteristics within and among males could mask the preferences for complex calls in the wild. In this study, we examine the acoustic preference of the midges using frogs calling at their breeding sites. Thus, we consider the effects of variation in call properties common in natural choruses at modulating midge attacks.

Our main goal in this study is to investigate calling properties of male túngara frogs in relationship to midge attraction. We hypothesize that call features that increase signal conspicuousness, such as call rate and call complexity, are important acoustic properties affecting midge attraction in their natural environment. To our knowledge, this is the first study to simultaneously quantify calling properties of male frogs as well as the attraction of eavesdropping insects in their natural habitat.

Methods

Study Site

The acoustic preference of frog-biting midges was investigated at three breeding sites surrounding the facilities of the Smithsonian Tropical Research Institute in Gamboa, Republic of Panama (9°07.0'N, 79°41.9'W). Gamboa is a small town that is surrounded by tropical rainforest as part of the Soberanía National Park. Túngara frogs breed in temporary puddles and ditches in and around Gamboa. At all breeding

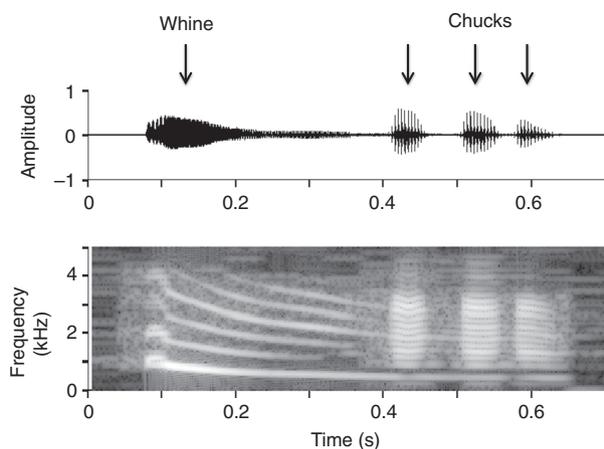


Fig. 1: Waveform and spectrogram of representative advertisement calls of túngara frogs. This call consists of a simple call, named 'whine', followed by secondary complex components named 'chucks'.

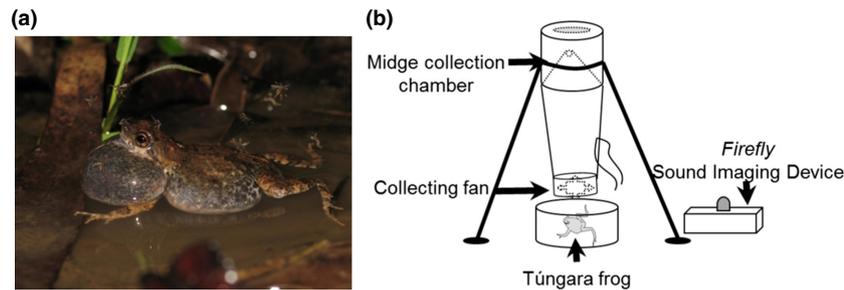


Fig. 2: Study species and recording setup used to investigate the behavior of túngara frogs and frog-biting midges in their natural habitat. (a) Calling male túngara frog attacked and bitten by frog-biting midges (photograph by X.E. Bernal). The midges are attracted to the frog's call and preferentially bite them on their nostril to obtain a blood meal. (b) Diagram illustrating the recording setup used in this study to quantify calling behavior and attraction of frog-biting midges to each focal male. The light patterns of deployed *Fireflies* were recorded by a video camera.

sites, we confirmed the presence of both túngara frogs and frog-biting midges prior to the recordings.

Field Recordings

Recordings were conducted during the rainy season between July and August, 2013. Between 20:30 and 0:00 hrs, chorus sizes of two or three were studied one at a time. At each chorus, a small round mesh cage (Diameter: 12 cm, Height: 7 cm) was placed on top of each frog to prevent him from moving away but still allowing him to vocalize normally (Fig. 2b). To investigate the calling behavior of the frogs, we used a sound imaging system based on individual devices, each called *Firefly*, that consists of a microphone and a light emitting diode (LED) activated by sound (Mizumoto et al. 2011). A *Firefly* was placed next to each cage containing a frog so the male's call would activate the LED of the device. The patterns of light activation produced by the *Fireflies*, as well as calls of túngara frogs, were recorded with a high-definition video camera (HDR-XR550V, Sony) for at least 15 min using 29.97 frames per second and 48 kHz sampling rate.

The LED of *Firefly* can be activated by the calls of several individual frogs if they are too close to each other (Mizumoto et al. 2011). Therefore, when additional frogs were found within close distance to focal individuals (<1.5 m) after placing the mesh cages, they were removed from the area prior to the beginning of the recordings.

To collect eavesdropping insects attracted to calling males, we used modified CDC miniature light traps following McKeever & Hartberg (1980) and Bernal et al. (2006). Each trap consisted of a small fan, without a light bulb, that was set on top of the cage of each calling male and blew approaching insects into a collection chamber (Fig. 2b). These traps successfully

collect frog-biting midges attracted to speakers broadcasting túngara frog calls (Bernal et al. 2006). The traps were placed on top of calling males, instead of a speaker in this study (at ~7 cm). Similarly to the playback experiments with speakers, any small insects approaching the call (in this case produced by the frog) were effectively collected by the traps. At the end of the recordings, we collected the chambers from the traps and inspected the cages and their surroundings for female túngara frogs. Time for collecting insects by the traps was synchronized with that for the video recording as precisely as possible. Then, the distances between pairs of neighboring males were measured and all males were captured, measured (snout-vent length and weight) and individually marked to recognize individuals. We marked the frogs by toe clipping them following the Guidelines for the Use of Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR). All frogs were then returned to the exact location where they were captured.

A total of 52 males were used in the field recordings but some of them escaped at the end of the recordings before they could be measured. The individuals used in this study were representative, in length (mean \pm SD = 27.9 ± 1.0 mm, $n = 48$ frogs) and weight (mean \pm SD = 1.79 ± 0.24 g, $n = 47$ frogs), of the local population (Ryan 1985). The recapture rate of the male frogs was 16.7% (8 of 48 individuals). The distance between pairs of neighboring males ranged at 1.5 m–14.8 m separation (mean \pm SD = 4.79 ± 3.52 m, $n = 34$ pairs).

To euthanize trapped insects collected by the traps on top of the calling males, all collection chambers were kept in the freezer for more than 8 hrs after the recordings. The specimens were then sorted and

preserved in 75% alcohol. We recorded the presence of blood meals (full or partial) across all specimens collected. Finally, frog-biting midges were identified to species following the key for Neotropical *Corethrella* by Borkent (2008). Given the early stages of the taxonomy of Corethrellidae, however, species names should be treated with caution.

Timing of Vocalizations

We estimated the timing of whines produced by túngara frogs by analyzing the light patterns of the sound-activated *Fireflies* following the methodology described in Mizumoto et al. (2011). The amplitude values of each pixel were summed across more than 500 frames of a video sequence and a subjective threshold was used to reduce the resulting image into a binary matrix that identified the position of each caller. By multiplying the binary matrix to each frame, the time series data of the light intensity of *Fireflies* were extracted. Here, the light intensity was calculated from RGB (red–green–blue) values at each pixel according to Mizumoto et al. (2011). Then, the onsets of the whines were detected as consecutive peaks of the light intensity exceeding a threshold value (Fig. 3) following Aihara et al. (2011). The responses of *Fireflies* are affected by multiple factors such as the sound pressure level of the calls and the distance from the frogs to the device. In addition, the distance from the *Fireflies* to the camera varied so that the intensity of LEDs captured by the camera had different dynamic ranges even in the same recording. To

precisely detect the onset of the whine from such diverse light patterns, for each recording we carefully chose the threshold value for the percentage of maximum light intensity in the analysis of each *Firefly* (35–70%). In some cases, there were frogs that did not call during the recordings. When there was such a silent frog in the vicinity of a *Firefly*, its LED was not activated and showed a very weak, noisy light pattern. Therefore, we distinguished silent frogs based on the light pattern of each device and described their call rates as 0 for the analysis.

Frog-biting Midge Attraction to Natural Call Rate and Complexity

Under simple, two stimuli conditions where call repetition rates are fixed at the same value, frog-biting midges are preferentially attracted to complex than to simple túngara frog calls (Bernal et al. 2006). This preference for calls with chucks parallels the preference of female túngara frogs (Rand & Ryan 1981) and frog-eating bats (Ryan et al. 1982). Here we investigate the responses of frog-biting midges to natural variation in call rate and call complexity in túngara frogs.

To detect complex calls, we compared the audio recording of the camera to the light pattern of each *Firefly*. This comparison is necessary given that the sound intensity of chucks is usually lower than that of whines, making it often impossible to detect the onset of chucks only from the light pattern in some recordings (Fig. S2 in Electronic Supplementary Material).

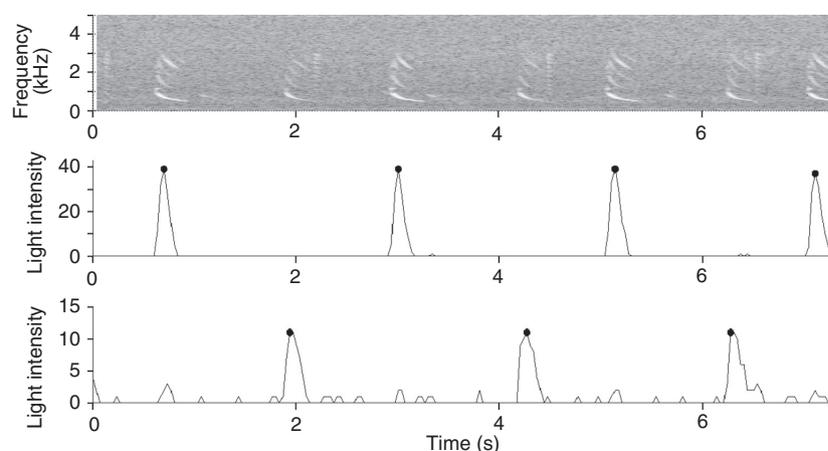


Fig. 3: Representative pattern of a túngara frog chorus detected during our recording. (Top panel) Spectrogram of the calls of túngara frogs. This spectrogram was obtained from the audio recorded by the video camera. (Middle and bottom panels) Light patterns of *Fireflies*. During this recording, two units of *Fireflies* were placed in the vicinity of the two chorusing frogs, one unit next to each frog. Solid lines represent the light patterns of the *Fireflies*, and filled circles represent call timings of the frogs that were estimated as the time of each light intensity peak. This figure illustrates how túngara frog calls were individually detected as different light patterns of the two *Fireflies* deployed.

Given that the signal-to-noise ratio in the audio recording varied to a great extent because of the variation in the number of other frogs calling in the same area, we carefully listened to the audio recording and checked its spectrogram to categorize the data of each recording as *noisy* or *clear*.

For the *noisy* cases ($n = 9$), we quantified the number of chucks only for the male frog nearest to the camera by carefully comparing the spectrogram to the light pattern of his *Firefly*. For the *clear* cases ($n = 9$), we quantified the number of chucks for all the focal frogs by comparing the spectrogram to the light patterns while carefully listening to the audio recording. The number of chucks produced by only one frog could not be estimated in one recording of the *clear* cases because his calls were not recorded with sufficient amplitude by a video camera. The number of chucks in each call was quantified for the first 30 calls of the focal frogs that did not overlap with the calls of other frogs. The number of chucks appended to a whine varied from 0 to 4, representing the natural variation in chuck production characteristic of this population (Bernal et al. 2007). The number of chucks produced per whine (i.e., number of chucks/30 whines, hereafter referred as chuck ratio) was then calculated for each frog ($n = 23$ frogs, 18 recordings).

We also examined the relationship between the call rate of individual frogs and the number of midges attracted to them. Call rate was calculated as the number of whines divided by recording time for each male frog. Here, recording time represents the whole time during which the camera was recording the illumination patterns of sound indication devices, which was synchronized with the time for the midge collection as precisely as possible. Overall, we treated frog-biting midges as a guild attacking túngara frogs and thus we pooled all *Corethrella* species together following Bernal et al. (2006).

Statistical Analyses

A generalized linear mixed model (GLMM) (Schall 1991) with a Poisson distribution and log link function was used to evaluate the effect of call rate and environmental factors (i.e., temperature and relative humidity) on the number of midges attacking the males. Because multicollinearity was confirmed in the relationship between temperature and humidity (Pearson's correlation coefficient: -0.5383 , temperature: 24.0 – 28.0°C , relative humidity: 63.5 – 76.5% , 18 nights), one of the environmental factors (relative humidity) was excluded from our analysis. Call rate and temperature were then treated as explanatory

variables describing fixed effects of the GLMM without any pairwise interaction terms. Study site was treated as an explanatory variable describing a random effect. Midge number was treated as the response variable. The significance of the fixed effects was estimated with Wald tests (Faraway 2006).

To further evaluate the role of call rate and how call complexity affects attraction of eavesdropper, additional GLMM was used. This separate analysis was necessary given we were able to collect data on call complexity only for a subset of the males. Multicollinearity between temperature and relative humidity was also confirmed in this subset (Pearson's correlation coefficient: -0.6270 , 15 nights), so that one of the environmental factors (relative humidity) was excluded from our analysis. While call rate, chuck ratio, and temperature were treated as fixed effects, study site was treated as a random effect. The pairwise interaction term between call rate and chuck ratio was also treated as a fixed effect in this analysis.

All the analyses were performed using R Statistical Software Version 3.1.1. The analyses with GLMMs were conducted using the system function '*glmmML*'. Throughout these analyses, recording time was treated as an offset term of the GLMMs.

Results

Imaging of Calling Behavior

The sound imaging system successfully captured the calling activity of túngara frogs in the chorus at their breeding aggregations. Figure 3 shows a representative recording illustrating how the calls of túngara frogs were individually detected as different light patterns from each *Firefly* device. During this recording, two units of *Fireflies* were placed in the vicinity of two chorusing frogs. The figure illustrates how the system matched the spectrogram of the túngara frog calls and the light patterns of the devices next to each individual.

In four of the 18 recordings, the device deployed in the vicinity of one of the focal frogs did not work properly. Two of the devices were too far from the camera and thus their lights could not be captured. One device responded to calls of a neighboring frog with similar light intensity to the response as to the calls of the focal male, making it impossible to selectively detect the onsets of calls of the focal frog. In the last case, the device showed different light intensity baselines during the same recording. Therefore, data from those four devices were excluded from the anal-

ysis. In addition, one of the fans from the midge traps accidentally stopped working on two nights. Therefore, the midge numbers obtained from those two traps were also excluded from the analysis on midge attraction.

Species of Frog-biting Midges

A total of 832 individual frog-biting midges representing six species were attracted to the frogs during our recordings. From those, *Corethrella squamifemora* was the most abundant species (82.81%), while *C. gloma* was the least abundant species (0.48%). Approximately 2% of the individuals captured were *C. peruviana*, while 1.2% was *C. quadrivittata*. A few individuals attracted to the frogs correspond to new, undescribed species (5.7% of *C. new sp 1* and 5.4% of *C. new sp 2*).

In our samples, some of the midges had a partial blood meal, indicating that the male that they were approaching was not their first victim that night. From the 832 midges collected, a total of 115 (14%) individuals had blood. Of the six species that we collected, however, only two species were observed to have blood (*C. squamifemora*: 111 individuals (96%); *C. peruviana*: 4 individuals (4%)). These findings are not surprising since male túngara frogs start to call at dusk, and the midges thus could have obtained a partial blood meal before the start of our recordings. Frog-biting midges attempting to feed from túngara frogs are often swat away by the frog shortly after initiating feeding. These interrupted feeding attempts result in partial blood meals as in some of the midges collected here, and make frog-biting midges able to act as vectors of disease.

Attraction to Frog Calls

Frog-biting midges were not equally distributed among the males in the chorus. The top panel of Table 1 shows the result of the GLMM evaluating the effect of call rate and temperature on the number of midges attracted by túngara frogs ($n = 46$ frogs). Male frogs calling at higher rates attracted a significant larger number of frog-biting midges ($\beta = 0.050 \pm 0.004$, $z = 11.440$, $p < 0.001$). Temperature also had a significant negative effect on the number of midges attracted ($\beta = -0.144 \pm 0.064$, $z = -2.242$, $p = 0.025$).

When considering call rate and chuck ratio, both call characteristics had significant positive effects on the number of midges attracted by the frogs (call rate: $\beta = 0.072 \pm 0.019$, $z = 3.754$, $p < 0.001$; chuck ratio:

Table 1: Summary of GLMM statistics for the effects of call properties and temperature on frog-biting midge attraction

Model	Fixed Factor	Estimates	SE	z	p
(i) Call rate	Call rate	0.050	0.004	11.440	<0.001
	Temperature	-0.144	0.064	-2.242	0.025
(ii) Call rate and complexity	Call rate	0.072	0.019	3.754	<0.001
	Chuck ratio	1.707	0.523	3.266	0.001
	Call rate x Chuck ratio	-0.074	0.023	-3.226	0.001
	Temperature	0.013	0.104	0.123	0.902

(i) The effect of call rate and temperature on midge attraction ($n = 46$).

(ii) The effect of call rate, chuck ratio, their pairwise term, and temperature on midge attraction ($n = 23$).

$\beta = 1.707 \pm 0.523$, $z = 3.266$, $p = 0.0011$). The bottom panel of Table 1 shows the result of the GLMM examining the effect of call rate, chuck ratio, their interaction term, and temperature on the number of midges attracted by túngara frogs ($n = 23$ frogs). The pairwise interaction term between call rate and chuck ratio had a significant negative effect ($\beta = -0.074 \pm 0.023$, $z = -3.226$, $p = 0.0013$). This interaction indicates that call rate and complexity do not have additive effects. Temperature did not have a significant effect on the number of midges attracted by the frogs ($\beta = 0.013 \pm 0.104$, $z = 0.123$, $p = 0.902$).

Female túngara frogs were found next to a calling male in three of the 18 recordings. In one case, however, the female was already in amplexus with other male. This amplexed pair likely came from outside of the study area because before the recording we had confirmed that no other male frogs were present in the area. These three females including one amplexed pair were found next to the male frog calling at the highest rate in the chorus, but the small sample size unfortunately precludes further analysis.

Discussion

Comparison of the light patterns, audio recordings, and the number of midges revealed that frogs calling at higher rates and producing more complex calls attracted more midges in their natural habitat. Two-speaker playback experiments with a fixed call rate have previously shown that frog-biting midges prefer complex to simple túngara frog calls (Bernal et al. 2006). Our results indicate that call rate is also an important predictor of frog-biting midge attraction to calling túngara frogs in nature. While several studies have shown that signaling rate affects female choice (reviewed in Ryan & Keddy-Hector 1992), fewer have

examined how it may affect attractiveness to eavesdroppers. Although not surprising, our results support the prediction that higher call rates are under selective pressures imposed both by sexual and natural selection.

The analysis using GLMMs demonstrated that both call rate and complexity play significant positive roles in midge attraction when those properties vary in natural choruses. We also found that the pairwise interaction term between call rate and chuck ratio had a significant negative effect. These results combined indicate that call rate and call complexity have similar impact on attractiveness to the midges. That is, the effect of call rate is particularly important in terms of midge attraction when the frogs are producing calls of lower complexity. Likewise, call complexity is especially important to predict the number of midges attracted when frogs call at low repetition rates. In addition, frogs producing calls of higher rate (or higher complexity) do not attract larger numbers of midges when they call at greater complexity (or greater rate). Environmental factors such as temperature may affect abundance of midges in the field site. However, we did not obtain the consistent results about the effect of temperature on the midge attraction: The analysis in the top panel of Table 1 shows a significant negative effect of temperature on the midge attraction, while the analysis in the bottom panel of Table 1 does not show a significant effect.

This study indicates that call rate and complexity are important properties of the calls influencing the responses of frog-biting midges. Other features of the calls (e.g., call intensity, dominant frequency, and frequency modulation), however, can also interact to shape attractiveness to eavesdroppers in similar ways as they do to female frogs. In particular, given that call intensity directly affects the active space of the signal, this trait is expected to increase signal attractiveness to both females and eavesdroppers. For instance, in the cricket *Eunemobius carolinus*, both relative and absolute intensities of the songs influence its attractiveness to conspecific females (Farris et al. 1997). Sound source localization and separation using a microphone array system (Grafe 1997; Simmons et al. 2008; Jones et al. 2014) would produce higher quality audio files that would allow detailed analysis of the acoustic properties of the calls to further investigate that possibility.

The temporal structure of a chorus results from strategies used by signaling males to increase mating opportunities while decreasing the chances of attacks by predators and parasites. In most anurans, neighboring males calling in a chorus alternate their calls

avoiding call overlap and thus reducing acoustic interference (Gerhardt & Huber 2002). Previous studies have described that male túngara frogs also vocalize alternating their calls with the calls of their neighbors (Ryan 1985; Greenfield & Rand 2000), which is consistent with our quantification of the timing of the calls in the chorus (Supplementary Material). Such call alternation between male frogs likely facilitates detection and localization by female frogs while also facilitating these tasks for eavesdroppers (Tuttle & Ryan 1982). In a few anuran species, males call in synchrony with neighboring males, a strategy expected to reduce attacks from eavesdroppers. In the cross-banded tree frog (*Smilisca sila*), for instance, males respond to the calls of neighboring males with extremely short latencies significantly overlapping their calls (Ryan 1986). This strategy of call synchronization has been proposed to result from strong selective pressures imposed by frog-eating bats (Tuttle & Ryan 1982). Further studies that directly examine the temporal structures of frog choruses in relationship to the responses of both intended and unintended receivers are necessary to better understand such trade-offs in anurans.

In this study, we limited the size of the choruses to a maximum of three individuals. Although male túngara frogs can form larger choruses, acoustic interactions between males are limited to a subset of close signaling neighbors, often attending to only the three closest ones (Greenfield & Rand 2000). It is possible that chorus size and its density affect long-distance midge attraction. In a larger chorus, for instance, more midges could be attracted due to a larger active space resulting from more males signaling together. Our study focused on the relative attraction of midges among up to three males calling in a chorus, but it likely represents the scenario encountered by subsets of interacting males that integrate larger choruses. In our sample, the chorus had a low male density. It is not uncommon to find túngara frogs calling close to neighboring males (<50 cm). Even though chorus density may affect the responses of frog-biting midges trying to bite neighboring males, our study represents the preferences of the midges after their first victim was chosen because they were captured at that point. Further studies that manipulate distance between males and its effects on midge attacks could provide valuable insights of potential strategies that males may use to minimize attacks by these parasites.

In our recordings, the LED of the *Firefly* was slightly illuminated even when there was no sound input (Mizumoto et al. 2011; Aihara et al. 2014). It is unlikely that the LED had an effect on the behavior of the

midges. Frog-biting midges rely exclusively on the advertisement call of their host without using other host-emitted cues often used by hematophagous insects such as carbon dioxide (Bernal & de Silva 2015). Although many insects are attracted to white light (Nabli et al. 1999), similar to blood-feeding mosquitoes (Bentley et al. 2009), frog-biting midges do not show this behavior and in fact respond by decreasing their movements in response to light (McMahon et al. unpub. data). In addition, the *Firefly* was deployed at approximately 10–20 cm from the cage containing the frog on top of which the fly trap was set, separating them in space to minimize potential interference of the LED and the midges. Similarly, the light was unlikely to affect the behavior of the frogs given that the *Firefly* is equipped with a red-colored LED that various anuran species do not see (Hailman & Jaeger 1974).

In conclusion, our study revealed that males calling at higher rates and with greater call complexity within the chorus attract a larger number of frog-biting midges. To our knowledge, this is the first study to simultaneously examine signaling strategies of male frogs as well as responses of eavesdropping insects in the wild.

Acknowledgments

We are grateful to M. Yasugi, M. Hoso, and T. Shimada for their valuable advice on our analysis with GLMMs. We are also grateful to H. G. Okuno for the support of the usage of a sound imaging system. The Smithsonian Tropical Research Institute provided logistic support that made this work possible. The Panamanian Environmental Agency, Autoridad Nacional del Medio Ambiente (ANAM), provided collection permits to perform this work (SE/A-64-13). This work was supported by a National Science Foundation grant (IOS-1258039/1433990) to X.E.B.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All procedures performed in this study were in accordance with the Guidelines for the Use of Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians

and Reptiles (SSAR). The field recordings performed in this study were approved by the Smithsonian Tropical Research Institute (IACUC# 2012-0712-2015).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1: Histogram of a phase difference Φ_{ij} between advertisement calls obtained from a single dataset of two chorusing frogs (those whose calling sequences are partially shown in Fig. 3).

Figure S2: Difficulty in estimation of complex calls just from light patterns.