



ORIGINAL ARTICLE

Within host acoustic signal preference of frog-biting mosquitoes (Diptera: Culicidae) and midges (Diptera: Corethrellidae) on Iriomote Island, Japan

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Abstract

Haematophagous insects can rely on specialized host-seeking behaviors to locate hosts. Some frog-biting flies, for example, eavesdrop on the conspicuous acoustic signals produced by male frogs and toads. Using such auditory cues to locate a host imposes an additional challenge: how to recognize appropriate sounds when different frog species produce calls with varying acoustic properties. The limited knowledge of antennal hearing in dipteran insects hinders our ability to understand how eavesdropping flies detect and recognize frog calls. Behavioral studies suggest that frog-biting flies use broad acoustic templates to detect and recognize their victims. Here, we use within-species call variation to examine the acoustic preferences in frog-biting flies. Specifically, we examine the attraction of frog-biting mosquitoes (*Uranotaenia* spp.) and midges (*Corethrella nippon*) to the calls of a Japanese treefrog, the Ryukyu Kajika frog (*Buergeria japonica*), on Iriomote Island, Japan. Male Ryukyu Kajika frogs produce two call types. While both calls have a high frequency peak (3 kHz), the first call type (Type I) also contains a lower frequency peak (1.8 kHz) absent in the second call type (Type II). Using field phonotaxis experiments we found that Type I calls are more attractive to both frog-biting mosquitoes and midges. Thus, our results suggest that the frog-biting Nematoceran flies in this community are biased towards the acoustic properties of Type I calls. We discuss this finding in the context of the evolution of antennal hearing in flies.

Key words: antennal hearing, *Buergeria japonica*, *Corethrella*, eavesdropping, frog-biting midges, *Uranotaenia*.

INTRODUCTION

Haematophagous insects rely on blood meals from hosts for reproduction and, in some cases, survival (Lehane 2005). There is thus strong selective pressure to locate appropriate hosts, which can result in specialized host-seeking behaviors involving a variety of sensory cues (Takken & Verhulst 2013). For example, some flies that bite frogs and toads, including mosquitoes of the genus *Uranotaenia* (Diptera: Culicidae) and midges of the genus *Corethrella* (Diptera: Corethrellidae), use auditory cues to locate their hosts

(e.g. McKeever 1977; Bernal *et al.* 2006; Borkent & Belton 2006). These frog-biting flies eavesdrop on the conspicuous advertisement calls produced by male frogs and toads. However, using auditory cues to locate hosts can be challenging. Unlike other host recognition cues, such as temperature and carbon dioxide, anuran mating calls are species specific with high diversity of spectral and temporal acoustic properties (Duellman & Trueb 1986).

Behavioral studies suggest that at least some species of frog-biting mosquitoes and midges have generalized acoustic templates that allow them to detect and localize the calls of multiple anuran host species (Virgo *et al.* 2019; Toma *et al.* 2019; but see de Silva *et al.* 2020). In some systems, frog-biting midges are attracted to a broad range of pure tones, 100–4000 Hz, and are preferentially attracted to tones around 2 kHz (Meuche *et al.* 2016). Pure tones, however, lack the

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acoustic complexity of frog calls. In general, frog-biting midges seem to prefer frog calls that are more acoustically complex, spectrally broadband, and lower frequency (<2 kHz), resulting in different eavesdropper attraction rates among host species (Grafe *et al.* 2019; Virgo *et al.* 2019) and among different call types within a given host (Bernal *et al.* 2006). Thus, while many eavesdroppers are attracted to the calls produced by multiple host species, not all calls are equally attractive to them. Even though the auditory mechanisms and sound perception by eavesdropping frog-biting flies are not fully understood (Page *et al.* 2014), their phonotactic responses in the field suggest their hearing system is biased towards certain host calls. Here, we examine this bias by conducting field phonotaxis experiments examining the responses of eavesdropping flies to call variation within a host species. Specifically, we investigate the attraction of frog-biting mosquitoes and midges to different call types of a Japanese treefrog, the Ryukyu Kajika frog (*Buergeria japonica*). By investigating the responses of frog-biting flies to the calls produced by individuals of the same frog species, functional explanations to potential call preferences, such as differences in defense strategies or blood quality, can be eliminated.

Male Ryukyu Kajika frogs produce two distinct call types (Fig. 1A,B). The social functions of either call type are unknown, although both seem involved in mate attraction (Tang 2009; Wang *et al.* 2017). Both call types are trills, containing notes repeated in a rapid redundant succession, and both are produced at variable durations, ranging from truncated calls of less than 0.5 s to longer calls over 3.0 s (Kuramoto 1986). The first call type (Type I), however, has low and high spectral frequency elements (dominant frequency at approximately 1.8 kHz and a co-dominant band at 3 kHz), while the second call type (Type II) has only a high frequency element (approximately 3 kHz). Given

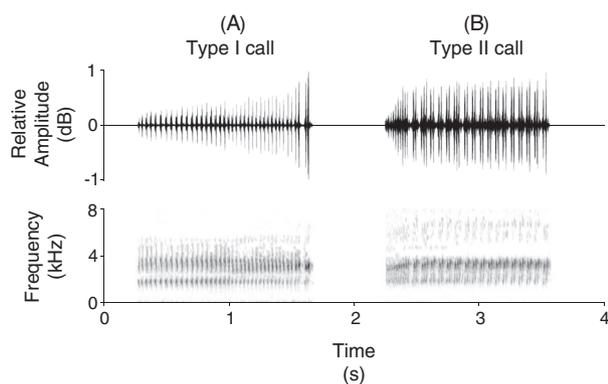


Figure 1 Oscillogram (top) and spectrogram (bottom) of a Type I (a) and Type II (b) Ryukyu Kajika frog call.

the difference in spectral frequency between the two frog call types, specifically that Type I calls have a wider spectral band and lower frequency elements, we predict that Type I calls are more attractive to eavesdropping flies than Type II calls.

MATERIALS AND METHODS

Eavesdropper selection for call type

To assess midge and mosquito attraction to the different call types of the Ryukyu Kajika frog, frog-biting insects were collected from 27 June to 25 July 2015 at six locations around Iriomote Island, Okinawa Prefecture, Japan (24°23'31.3"N 123°52'46.6"E). All locations were greater than 1 km apart (following Legett *et al.* 2019) and chosen to cover a range of habitats in which Ryukyu Kajika frog choruses occur; two in undeveloped forest (outside of the state protected zone), two near rice paddy fields, and two along roadside ditches. These locations were each near a water source with breeding frogs, but not close to large frog choruses, waterfalls, or other sources of intense environmental noise. Each of the six locations was sampled five times over the collection period for a total of 30 trials. Eavesdropping insects were collected using modified CDC mosquito traps (McKeever & Hartberg 1980) placed over speakers (Pignose Model 7–100) following well-established, standard procedures (e.g. Bernal *et al.* 2006). Within a location, two speakers with traps were placed 30 m apart. One speaker broadcast Type I calls while the other broadcast Type II calls. Broadcast calls were haphazardly chosen from a library of 10 pre-recorded calls of each type. All calls in this library were recorded from different male frogs. Both call types were broadcast at a rate of one call every 2 s at an amplitude of 80 dB SPL re. 20 μ Pa (Brüel & Kjær digital sound level meter Type 2250) measured from 1 m at ground level. While the natural call rate and amplitude of Type I and Type II calls can differ, our goal was to investigate only the effect of call structure.

At a location, insects were collected for 45 min starting 30 min after sunset (Legett *et al.* 2019). After collection, all insects were killed overnight in a freezer. The following day, all the insects were counted and stored in 75% ethanol for species identification. Established keys were used to identify eavesdroppers to species, *Uranotaenia* (Miyagi & Toma 2013) and *Corethrella* (Borkent 2008). Since some moth flies of the genus *Sycorax* (Family: Psychodidae) are known to eavesdrop on frog calls (Cutajar & Rowley 2020), we also counted and separated moth flies in our collection.

Moth flies captured here, however, were not of the frog-biting genus *Sycorax* (Quate 1966) and were instead in the genus *Psychoda*, a group not associated with frogs, and known instead for their role in decomposition and pollination (Byrd & Castner 2001; Johnson & Schiestl 2016).

Statistical analysis

Statistical analyses were conducted using Program R v. 3.5.2 (R Development Core Team: www.R-project.org). The difference in attraction of insects to each Ryukyu Kajika frog call type was analyzed using the generalized linear mixed effect model (GLMM) function in the *glmmTMB* package (Brooks *et al.* 2017). Call type (I or II) was included as a fixed factor, location as a random factor and date as a random factor nested within location. In some cases, multiple trials were performed at different locations on the same night, so trial number was also included as a random factor nested within date to account for potential effects of sampling time over the course of each night. The specific playback used for each trial (playback ID) was included as an additional, non-nested, random factor.

For the three groups of frog-biting insects (mosquitoes, midges, and potentially moth flies), GLMMs with Poisson distributions and log link functions were used to compare eavesdropper attraction between call types. Zero-inflated models were used for the mosquito and midge models, and a non-zero-inflated model was used for the moth flies. Differences in the number of individuals collected as bycatch, non-frog-biting insects, were analyzed using a GLMM with a negative binomial II

distribution and a log link function. All models were fit based on Akaike information criteria (AIC; following procedures in Bolker *et al.* 2009) and evaluated using the model diagnostic R package *DHARMA* (Hartig 2018). Assumptions of homoscedasticity were additionally tested using Levene’s tests in the *car* package (Fox & Weisberg 2019). Differences in the least squares means obtained from the models were used as estimates of effect sizes.

RESULTS

Frog call traps broadcasting Ryukyu Kajika frog calls successfully captured frog-biting mosquitoes (n = 100), midges (n = 74), and moth flies (n = 37), as well as numerous non-frog-biting insects (Table 1). Four frog-biting mosquito species were identified, two from the subgenus *Pseudoficalibia* (*Ur. nivipleura*, *Ur. ohamai*) and two from the subgenus *Uranotaenia* (*Ur. annandalei*, *Ur. macfarlanei*). Of these, *Ur. macfarlanei* was the dominant species, representing 71% of the mosquitoes captured. Significantly more frog-biting mosquitoes were captured using Type I calls, which attracted 2.80 ± 0.85 (mean \pm standard error) mosquitoes per 45 min trial compared to 0.57 ± 0.16 mosquitoes for Type II calls ($z(53) = 4.25$, $P < 0.001$, effect size = 1.66; Fig. 2A). Similarly, Type I calls were also significantly more attractive to frog-biting midges, attracting 2.00 ± 1.06 midges per 45 min trial compared to 0.47 ± 0.22 midges for Type II calls ($z(53) = 2.80$, $P = 0.005$, effect size = 1.37; Fig. 2B). All captured frog-biting midges were of the same species, *Corethrella nippon*. In contrast to frog-biting mosquitoes and midges, the number of *Psychoda* moth flies

Table 1 Numbers of insects collected by frog call traps broadcasting either type I or Type II Ryukyu Kajika frog calls

Family	Species	Number of insects attracted to calls			z-value	P-value	effect size
		Type I	Type II				
Culicidae	<i>Ur. (Pseudoficalibia)</i>						
	<i>nivipleura</i>	1	0	-	-	-	
	<i>ohamai</i>	6	5	-	-	-	
	<i>Ur. (Ura.)</i>						
	<i>annandalei</i>	12	5	-	-	-	
	<i>macfarlanei</i>	65	6	5.34	< 0.001*	2.56	
	Total <i>Ur.</i>	84	16	4.25	< 0.001*	1.66	
Corethrellidae	<i>Corethrella nippon</i>	60	14	2.80	0.005*	1.37	
Psychodidae	<i>Psychoda</i>						
	spp.	22	15	1.14	0.253	0.38	
Bycatch		177	257	-0.90	0.374	-0.19	

Mosquitoes (Culicidae) and midges (Corethrellidae) were analyzed using generalized linear mixed effect models (GLMMs) with zero-inflated Poisson error structures, moth flies (Psychodidae) were analyzed using a GLMM with a non-zero-inflated error structure, and non-eavesdropping insects (Bycatch) were analyzed using a GLMM with a negative binomial II error structure (n = 30 nights, df = 53). Statistically significant differences ($P < 0.05$) are indicated with a “*”.

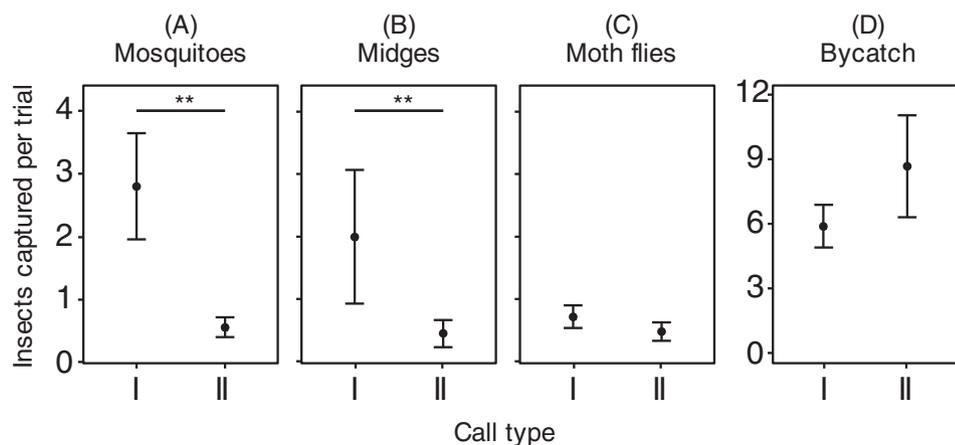


Figure 2 Frog-biting mosquitoes of the genus *Uranotaenia* (a), frog-biting midges of the genus *Corethrella* (b), moth flies (c), and bycatch (d) attracted to either Type I or Type II Ryukyu Kajika frog calls. Values are means and standard errors. Statistically significant differences between call types ($P < 0.05$) are indicated with a “*”. Note the difference in scale for bycatch.

captured did not significantly differ between call types ($z(53) = 1.14$, $P = 0.253$, effect size = 0.38; Fig. 2C). There was also no significant difference in the number of non-frog-biting insects captured between call types ($z(53) = -0.90$, $P = 0.374$, effect size = -0.19 ; Fig. 2D). The number of insects collected for each sampling location is included in the online Supporting Information section (Tables S1 ad S2).

DISCUSSION

We found that the two Ryukyu Kajika frog call types differ in their attraction of eavesdropping flies. As expected, the call type with additional lower frequency element (Type I calls) attracted significantly more frog-biting mosquitoes (*Uranotaenia* spp.) and midges (*Corethrella nippon*). This finding is consistent with the preferences of frog-biting midges found in other systems, where frog species with lower frequency calls (<2 kHz) attract more midges than frog species with higher frequency calls (Grafe *et al.* 2019; Virgo *et al.* 2019). A similar pattern in preference among the calls of different host species has also been shown with frog-biting mosquitoes in Japan (Toma *et al.* 2019). The most abundant frog species with a low frequency call was generally the most attractive to all eavesdroppers (*Hylarana glandulosa*: Grafe *et al.* 2019; *Fejervarya sakishimensis*: Toma *et al.* 2019; *Leptodactylus savagei*: Virgo *et al.* 2019). These studies, however, relied on inter-species variation in call structure. Thus, these previous studies potentially confound variation in call acoustics with trade-offs associated with biting different species. Our results, revealing differences in eavesdropper attraction rates among call types within a host species, support the hypothesis that “low” frequency components increase attraction to frog-biting flies and rule out alternative explanations

arising from differences among host species. These within host findings are consistent with frog-biting midge preferences for the calls of túngara frogs (*Engystomops pustulosus*), where complex calls, which have additional energy at low frequencies, are more attractive than simple call types (Bernal *et al.* 2006). The difference in amount of energy at low frequencies, however, is not the only difference among preferred and less-preferred calls. The complex calls of túngara frogs and Type I calls of Ryukyu Kajika frog share yet another feature, they both have multiple frequency peaks. In sum, the phonotactic behavior of frog-biting flies tested here and in other systems worldwide suggests their differential attractiveness to frog calls is due to generalized acoustic templates biasing lower frequency sounds, sounds with a complex frequency spectrum, or both.

Despite the independent evolutionary origins of eavesdropping on frog calls (Wood & Borkent 1989), frog-biting mosquitoes and midges share their preferences for the same call features. Given that flies from both lineages use antennal hearing, this preference likely reflects sensory biases of this hearing organ. Antennal hearing has received great attention in a mating context in mosquitoes (Gopfert *et al.* 1999; Nadrowski *et al.* 2011; Albert & Kozlov 2016), but less is known about how these particle detection organs would detect frog calls (Page *et al.* 2014). It has long been assumed, for instance, that antennal hearing was restricted to sounds at close distances (Gopfert & Robert 2001), questioning how frog-biting mosquitoes and midges could hear and phonotax to frog calls from a distance. Recent studies, however, indicate that the antennae of mosquitoes can detect sound sources farther away than previously predicted (Menda *et al.* 2019). As the use of acoustic signals to find hosts may have evolved from using sound in a mating

context (de Silva *et al.* 2015), additional adaptations that facilitate detecting frog calls are expected to have evolved in frog-biting mosquitoes and midges. Such adaptations in antennal hearing, however, would have evolved under limitations imposed by morphological and physiological constraints (Lapshin & Vorontsov 2017; Römer 2020), potentially resulting in the biases highlighted in this study. The acoustic preferences of frog-biting flies, as shown here and in other studies, suggest that these preferences may result from a sensory bias for low frequency sounds, distortion products produced by the interaction of multiple frequency peaks, or both. Further investigations of the biomechanical properties and auditory physiology of antennae of frog-biting flies are needed to better understand why general acoustic preferences seem to hold across eavesdropping frog-biting mosquito and midge species.

In contrast to frog-biting mosquitoes and midges, the moth flies captured in our study did not display a bias for either call type. The number of individual moth flies captured is comparable to those of other frog-biting species collected here, which may suggest the use of acoustic cues rather than random bycatch. A previous collection using acoustic frog call traps on Iriomote Island reported capturing moth flies at rates similar to those found in this study (Toma *et al.* 2005). That study, however, found no difference between traps broadcasting frog calls and silent, control traps suggesting that moth flies in this system are not eavesdropping. Frog-biting moth flies from the genus *Sycorax* eavesdrop on frog calls in Australia (Cutajar & Rowley 2020), but we did not capture species from this genus here. Furthermore, in other parts of the world *Sycorax* spp. do not seem to rely on host-emitted calls to bite frogs (Bravo & Salazar-Valeznuela 2009; Mačát *et al.* 2015). The prevalence of eavesdropping in other moth fly genera is unknown.

Finally, attracting eavesdropping flies can be costly for hosts due to blood loss (Camp 2006) and disease transmission (Johnson *et al.* 1993; Bernal & Pinto 2016). Thus, differential attraction of eavesdropping frog-biting insects is likely a driver of signal evolution among frogs and toads. Call variation and eavesdropper avoidance behaviors, in particular, can be impacted by selective pressures imposed by eavesdroppers (Legett *et al.* 2019). Examples of such avoidance behaviors in frogs include calling in habitats with high levels of abiotic background noise and calling in synchrony with other frogs in the chorus (Tuttle & Ryan 1982; Legett *et al.* 2019). Similarly, eavesdroppers may impose different selective pressure among different

call types within a species (Bernal *et al.* 2006). Our results suggest that, given eavesdropper bias for Type I calls, the cost of producing Type I calls may be greater than Type II calls for male Ryukyu Kajika frogs. However, to examine how eavesdropper selection may have shaped signaling behavior in Ryukyu Kajika frogs, the attractiveness of each call type to female Ryukyu Kajika frogs must also be considered. While previous studies suggest that both call types may attract mates (Tang 2009; Wang *et al.* 2017), their relative attraction and exact social function is unknown. Future studies examining tradeoffs in the attraction of mates and eavesdropping flies in this system might prove valuable in understanding the role that frog-biting mosquitoes and midges play in signal repertoire evolution.

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SUPPORTING INFORMATION

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

Table S1. Numbers of insects collected by frog call traps broadcasting Type I Ryukyu Kajika frog calls at each of the six sampling locations. Each location was sampled five times over the collection period for a total of 30 trials.

Table S2. Numbers of insects collected by frog call traps broadcasting Type II Ryukyu Kajika frog calls at each of the six sampling locations. Each location was sampled five times over the collection period for a total of 30 trials.