Cues used in host-seeking behavior by frog-biting midges (*Corethrella* spp. Coquillet)

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ABSTRACT: We investigated the role of carbon dioxide and host temperature in host attraction in frog-biting midges (*Corethrella* spp). In these midges, females are known to use frog calls to localize their host, but the role of other host-emitted cues has yet not been investigated. We hypothesized that carbon dioxide acts as a supplemental cue to frog calls. To test this hypothesis, we determined the responses of the midges to carbon dioxide, frog calls, and both cues. A significantly lower number of midges are attracted to carbon dioxide and silent traps than to traps broadcasting frog calls. Adding carbon dioxide to the calls does not increase the attractiveness to the midges. Instead, carbon dioxide can have deterrent effects on frog-biting midges. Temperature of calling frogs is not a cue potentially available to the midges. Contrary to our hypothesis, there was no supplemental effect of carbon dioxide when presented in conjunction to calls. Midge host-seeking behavior strongly depends on the mating calls emitted by their anuran host. Overall, non-acoustic cues such as host body temperature and carbon dioxide are not important in long-distance host location by frog-biting midges. *Journal of Vector Ecology* **40 (1): 122-128. 2015.**

INTRODUCTION

A critical challenge encountered by hematophagous insects involves locating an appropriate host to obtain a blood meal. For permanent ectoparasites, once they find a host, they enjoy a long-lasting supply of food. Many temporary ectoparasites, however, depend on seeking a host multiple times during their lifetime. Accordingly, hematophagous insects that do not remain on their host have sophisticated adaptations to efficiently search and find their victims. For instance, host-seeking malaria mosquitoes (*Anopheles gambiae* Giles *sensu stricto*) that exhibit a strong preference towards human hosts have specialized receptors that detect particular human foot odors that are enhanced as a result of *Coryneform* bacterial growth (Braks et al. 2000). Adaptations that increase long-distance host detection and localization are present across blood-sucking insects allowing them to efficiently find their host.

A variety of host-emitted cues are used by hematophagous insects to detect their host. Olfaction, however, has been recognized as the most widespread sensory modality used among hematophagous insects to find their host (Bowen 1991, Davies 1996, Gillies 1980, Zwiebel and Takken 2004). Many studies have thus focused on investigating odor-mediated host-seeking behavior. In mosquitoes, which are of great medical importance, a variety of chemical compounds are recognized to determine orientation towards their hosts. Among those compounds, carbon dioxide is the host-emitted cue that has received the most attention in host-seeking behavior, given its high effectiveness at attracting mosquitoes both in field and laboratory conditions (Bowen 1991, Gillies 1980, Kline 1994, Kline et al. 1990). Indeed, many species of mosquitoes have particular adaptations to detect carbon dioxide emitted by their hosts. Electrophysiological recordings from carbon dioxide receptors on the palps of *Aedes aegypti*, for instance, revealed their ability to respond to particularly low concentrations of carbon dioxide (150-300 ppm) and are stimulated by changes in concentration as slight as 0.01% (Grant et al. 1995). Given that traps with intact hosts are usually more effective than traps baited with carbon dioxide only, however, other cues are also probably used in conjunction with carbon dioxide by many species. For certain mosquito species, for example, octenol is an effective attractant alone or in combination with carbon dioxide (Takken and Kline 1989, Kline et al. 1991, Hoel et al. 2007). Similarly, lactic acid can also increase trap effectiveness when combined with carbon dioxide (Kline et al. 1990). In mosquitoes, exploitation of such robust host-seeking behavior has led to the implementation of chemical control to manage their populations. Although extensive work has investigated host-seeking behavior and, in particular, the cues used by mosquitoes to find a host, in comparison little is known about such behavior and cues in closely related families. Here we investigate long-distance cues used in host-seeking behavior by frog-biting midges (*Corethrella* spp Coquillet), the sister taxa to phantom midges (Chaoboridae) and mosquitoes (Culicidae).

Frog-biting midges are a tropical and subtropical family of small flies that consist of 105 recognized, extant species (Robert and Borkent 2014). In this monogeneric family, females feed from blood from anurans and, as in many female mosquito species, they depend on a blood meal for egg production. In contrast to most mosquitoes, however, female frog-biting midges use the mating calls of their host to locate their victim. Attracted by the mating call of frogs and toads, female midges approach their host landing directly on the frog or on vegetation close-by from where they walk seeking an opportunity to bite. Although anuran mating calls are sufficient to attract frog-biting midges (McKeever 1977,

Figure 1. Frog-biting midges attacking a calling male túngara frog. Photo by Ryan Taylor.

Borkent 2008, Bernal et al. 2006), it is unknown whether other host-emitted cues are also used in finding their host. Here we investigate the role of host-emitted cues in host-seeking behavior in frog-biting midges in the field, focusing on their interactions with their main anuran host at our study population in Panama. At this site, frog-biting midges are attracted to the mating call of túngara frogs (*Engystomops pustulosus*) in large numbers (Figure 1). The midges are vectors of blood parasites (Johnson et al. 1993) and in túngara frogs, they infect them with trypanosomes (Bernal and Pinto, unpublished data). Although the acoustic stimulus alone is sufficient to elicit host-seeking behavior (Bernal et al. 2006), it is unknown whether other host-emitted cues are used by the midges to effectively detect and localize their hosts.

In this study, we examined the use of non-acoustic cues by frog-biting midges. We focused particularly on the potential role of carbon dioxide in providing host cues given the widespread effect of this chemical compound in host-seeking behavior in blood-sucking insects (Nicolas and Sillans 1989). Since the temperature of the host has also been recognized as an important host-emitted cue used by mosquitoes (Daykin et al. 1965, Khan and Maibach 1971, Parker 1952, Willis 1947), and some mosquito species have the ability to perceive minute temperature gradients (Khan et al. 1966), we evaluated whether frog body temperature could provide information about host location to the midges. Although vertebrate ectotherms usually rely on external heat sources to modulate their body temperature (Huey 1982, Hutchinson and Dupré 1992), calling male frogs can have elevated temperatures compared to their surrounding environment (Passmore and Malherbe 1985). Finally, we took the first steps at investigating the role of chemical cues such as skin peptides in promoting host-seeking behavior in frog-biting midges. We hypothesized that cues additional to the mating call of frogs provide supplemental cues that are used by frog-biting midges for host location.

MATERIALS AND METHODS

Study area

Frog-biting midges were collected during the rainy season in the vicinity of anuran breeding ponds around the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07.0'N, 79°41.9'W). This area is surrounded by the National Park Soberania, a low-land rainforest that is located along the eastern edge of the Panama Canal. We performed experiments to investigate the host-emitted cues used by frog-biting midges between 2012 and 2014.

Host temperature and presence

To measure the temperature of calling males and their immediate surrounding environment, we used a MT6 Raytek MiniTemp hand-held non-contact, infrared thermometer. We sampled 25 mm^2 areas directly on the thoracic dorsum of the calling male and at four areas, each at 10 cm from the frog. We chose those four locations based on a Cartesian coordinate system where the frog was located at the origin, with its vertebral column along the y-axis. Individuals calling from a variety of túngara frog breeding habitats in the field were sampled (N=30).

To investigate the role of non-acoustic cues, we placed silent túngara frogs by a calling individual and counted the number of midges attracted to each individual after 30 min. Female túngara frogs, as most female anurans (Schalepfer and Figueroa-Sandí 1998), do not produce mating calls (Ryan 1985a). We thus captured a female and placed her under a mesh cage with holes large enough for the midges to reach the frog inside but too small for the female to escape. One female inside her cage was placed 50 cm from a calling male, also inside a cage identical to the one used for females. Once the male reassumed calling after being disturbed, we waited 30 min and, at the end of that time, counted the number of frog-biting midges on both frogs inside their cages. By using a silent frog as a lure, we examined the potential role of skin peptides as well as carbon dioxide production by the host in long distance host-seeking behavior.

Acoustic stimulus

The mating call of túngara frogs consists of a low-frequency whine with a fundamental frequency that sweeps from 1,000 to 500 Hz in 300 msec (Rand and Ryan 1981). When males acoustically interact with other males, they add short secondary components ("chucks") to their call (Ryan 1985a), appending up to seven chucks to a single whine (Bernal et al. 2007). While complex calls are more attractive to females, they also increase the chances of attack by eavesdropping predators and parasites (frog-eating bat, *Trachops cirrhosus*, Tuttle and Ryan 1981; frog-biting midges, Bernal et al. 2006). Frog-biting midges come to the frogs attracted by their mating call, land on the frog's thoracic dorsum and then walk towards the head, ultimately biting túngara frogs at the nostril. This bite site selection increases blood-feeding efficiency by the midges given the skin characteristics of túngara frogs (de Silva et al. 2014). To evaluate midge presence and examine potential additive effects of non-acoustic cues paired with mating calls, we used a synthetic version of a túngara frog whine either alone or in conjunction with carbon dioxide. The acoustic stimulus was synthetized based on the mean values of the parameters of the mating calls from 50 males in the study population. We used the software developed by J. Schwartz (Pace University at Pleasantville, NY; sample rate 20 kHz and 8 bit) to shape sine waves following the mean call parameters estimated before. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003).

Carbon dioxide experiments

To examine the role of carbon dioxide on host-seeking behavior we used several sources of this chemical compound as attractants. In all cases, however, we used the same trapping technique. Modified CDC miniature light traps following Bernal et al. (2006), with no light bulb, were used with carbon dioxide, a mating call, and/or a túngara frog as attractants. In all cases, a speaker (Pignose Model 7-100; Pignose-Gorilla, North Las Vegas, NV) was placed under the collecting fan of the trap (even if kept silent). The traps were opened for 20-45 min depending on the experiments and after that time, the collection chambers were frozen to euthanize the insects and store them in 75% ethanol. Frog-biting midges (*Corethrella* spp) from each trap were counted to calculate the attractiveness of the lures used at each trap. Samples from 2012 and 2013 were identified following Borkent (2008). Given the early stages of the taxonomy of Corethrellidae, however, species names should be treated with caution.

Since carbon dioxide traps using dry ice are an effective trapping technique commonly used with mosquitoes (Defoliart and Morris 1967, Theimann et al. 2001, Silver 2008), we performed preliminary trials implementing this technique. We used 5 lbs of dry ice that were insulated in dry ice buckets for EVS CO2 traps (BioQuip Inc). Small tubing (0.5 cm in diameter) released carbon dioxide on top of the speaker placed below the trap. To evaluate whether carbon dioxide is sufficient to attract frog-biting midges, we set-up independent traps releasing carbon dioxide with no other stimuli. While these traps with dry ice attracted high numbers of mosquitoes (>700 individuals, *Armigeres* and *Culex* species), they did not capture any frog-biting midges, even though these midges were observed attacking frogs in the same breeding area where the trap was located.

To further examine this phenomenon, we deployed three traps with different attractants, located >3 m apart from each other along an anuran breeding area. One trap released carbon dioxide only (2.2 kg dry ice), a second trap broadcast a túngara frog whine, and the third trap contained a frog inside a mesh cage. Treatments were rotated through all three traps so a given attractant was not used in the same trap in consecutive trials. All trials were performed in different nights to prevent carry-over effects from one treatment to the other.

To further investigate whether carbon dioxide is used in host-seeking behavior by frog-biting midges, we used a 3.1 kg carbon dioxide tank with a Western Medica MI-540-P regulator with a FME 0107 flow meter that allowed us to control the rate of gas emission at the traps. We used an emission carbon dioxide rate of 500 ml/min, which is within the range of CO_2 rates used in previous studies with several species of mosquitoes and equivalent to the rate used by commercial mosquito traps (Dennett et al. 2004, Farajollahi et al. 2009, Meeraus et al. 2008). Using this improved carbon dioxide emission system, we set up one trap with carbon dioxide only as the stimulus and a second trap broadcasting the túngara frog whine. The traps were placed 3-4 m apart from each other and were opened for 20 min. Having a trap broadcasting the mating call allowed us to examine the presence and abundance of frog-biting midges only in the trapping area.

To determine whether there is an additive effect of carbon dioxide when combined with the mating call, we performed two types of experiments. Although the source of carbon dioxide was different in the experiments, in both cases we used a trap broadcasting the túngara frog whine (call only) and a second trap releasing carbon dioxide as well as broadcasting the mating call (call + CO_2). In the first experiment, we used the CO_2 tank releasing carbon dioxide at a rate of 500 ml/min. To eliminate concerns about the elevated rates of carbon dioxide emitted by traps with the CO_2 tank compared to those produced by anurans, we performed a second experiment using a Z-SeriesTM Capsule Activation (ICA TriNova LLC) modified to release extremely low carbon dioxide emissions, at a rate of ~ 1 ml/min.

RESULTS

Seven species were attracted to the traps set up in this study (Table 1). From those, *Corethrella squamifemora* Borkent was the most abundant species (2013 samples: 40% for call only traps and 42% for call + CO_2 traps; in 2012 samples: 68% for sound only and 53% for sound +CO_2), while *C. quadrivitata* Shannon was the least abundant species. A few individuals attracted to the traps correspond to a new, undescribed species. The same species were captured in traps from different treatments and years.

Host temperature and presence

The temperature of calling túngara frogs was not significantly different from its immediate surrounding environment (Wilcoxon Signed Rank Test, Z=-0.794, p=0.427, N=30). In all cases, the temperature of the frogs was the same as at least one of the environmental points measured at 10 cm from it, and no more than 1° C from the average temperature of the environment. Indeed, the temperature of calling frogs (mean± SE: 26.1±0.2° C) was almost identical to the average temperature of its surrounding

Table 1. Proportion of specimens from each species of frog-biting midges (*Corethrella* spp) collected from traps using túngara frog mating calls and/or carbon dioxide. "Damaged" individuals includes those specimens that were in poor shape for identification to the species level but were confirmed to be *Corethrella*.

Species name	Percentage of specimens from each species collected in each trap			
	2012		2013	
	Sound alone	Sound+CO_2	Sound alone	Sound+CO_2
C. quadrivitata Shannon	0.8	2.0	1.6	1.4
C. gloma Borkent	2.6	1.7	12.5	9.2
C.longituba Belkin	1.9	1.3	2.7	4.3
C. peruviana Lane	2.2	4.4	6.5	4.7
C. squamifemora Borkent	68.2	52.1	39.6	41.7
C. new species 1	2.6	9.8	16.1	14.5
C. new species 2	2.2	6.3	9.6	9.9
Damaged individuals	19.5	22.3	11.4	14.4

environment (26.0±0.21° C). In addition, there was no detectable effect of a silent frog at attracting midges. Silent túngara frogs attracted fewer midges than a calling frog located just a short distance away (Wilcoxon Signed Rank Test, Z= 3.437, p=0.001, N=15). While females only were attacked by a frog-biting midge in rare occasions (two out of 15), in those cases they were attacked by a single midge. In contrast, all calling males attracted frog-biting midges and half of them attracted at least three midges in half an hour (eight out of 15), with some males having up to seven midges attacking them at the end of the experiment.

Carbon dioxide

Traps using carbon dioxide as a lure, independently of the release rate, failed to attract frog-biting midges. When using dry ice traps as the source of carbon dioxide, not a single frog-biting midge was attracted to the trap emitting carbon dioxide. Similarly, traps with a silent but live frog attracted almost no midges. Overall, traps with CO_2 or a silent frog attracted a significantly lower number of midges than the trap broadcasting the mating call only (Figure 2, Kruskal-Wallis Test statistic = 12.091, p= 0.002, df=2). In agreement with these findings, traps using the lower carbon dioxide emission rate released from the CO_2 tank revealed that a significantly higher number of midges were attracted to trap broadcasting the mating call only than a trap with carbon dioxide only (Figure 3a. Wilcoxon Signed Rank Test, Z= -2.023, p=0.043, N=5 nights).

Contrary to our expectation, there was not an additive effect of having the mating call together with carbon dioxide at a trap. Indeed, the effect was either the opposite or absent. When we used a CO_2 tank to released carbon dioxide (500 ml/min), a significantly lower number of frog-biting midges were attracted to a trap broadcasting the mating call and releasing CO_2 than a trap with the mating call only (Figure 3b. Wilcoxon Signed Rank Test, Z= -2.805, p=0.005, N=10). With a lower emission rate (1 ml/min), however, the deterrent effect of carbon dioxide was not observed (Figure 3c, Wilcoxon Signed Rank Test, Z= 0, p=1, N=10).

DISCUSSION

Overall, our results showed that túngara frog mating calls are not only sufficient to attract frog-biting midges but are also necessary to elicit host-seeking behavior in these eavesdroppers. None of the other potential host-emitted cues evaluated in this study were successful at attracting the midges when presented without mating calls.

Calling túngara frogs sustain long periods of vocalization production (three to four hours a night), a process that is energetically demanding compared to all other activities performed by the frogs. Similar to other acoustic animals, túngara frogs have very low energetic efficiency when calling (0.5-1.2%, Ryan 1985b). It would thus be possible that this effort could raise the temperature of calling males above the environment. In painted reed frogs (*Hyperolius marmoratus*), for instance, calling males have elevated temperatures that depart from environmental temperatures (Passmore and Malherbe 1985). Contrary to the findings in this arboreal species of frogs, however, our measurements show that the temperature of calling túngara frogs is not a cue available for frog-biting midges to differentiate their host from their environment and detect the presence of the calling frog. Túngara frogs call in water, a medium that given its high specific heat capacity may reduce an increase in body temperature due to calling effort. Although host temperature is an important host-seeking cue used by most hematophagous insects (Davis and Friend 1995), the role of this cue may be more commonly used by parasites feeding on endothermic hosts.

Other non-acoustic cues are probably not important in host-seeking behavior in frog-biting midges. In general, traps using intact hosts to lure hematophagous insects are more efficient than those using a single or few host-emitted cues. Frog-biting midges, however, were not attracted to silent but otherwise intact, túngara frogs. Instead of using males, the usual victim of the midges, in our experiment we used female frogs given their inability to vocalize. Since frog-biting midges readily bite female túngara frogs that are placed on top of a speaker broadcasting túngara frog calls
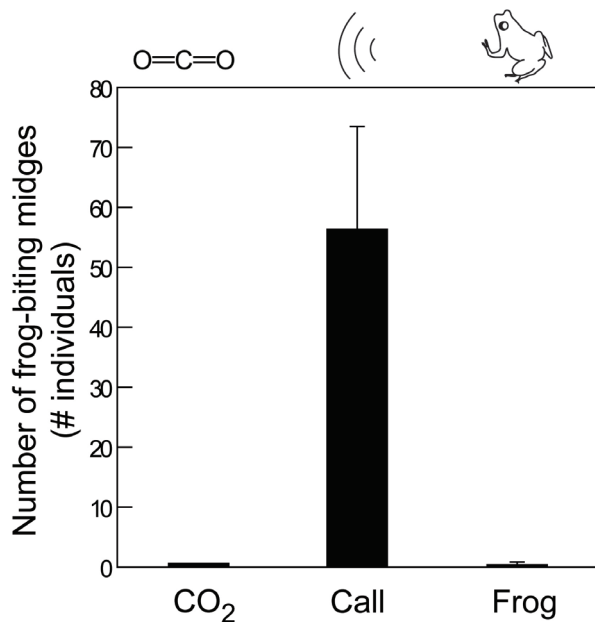
Figure 2. Frog-biting midges attracted to traps emitting a variety of host cues. In this experiment, carbon dioxide was released using dry ice.

(Bernal, *unpub. data*), it is highly unlikely that our results are due to sexual differences in attractiveness of the frogs. In addition, in some occasions, frog-biting midges attacking a male in amplexus move from the nostril of the male to the nostril of the female and bite her (Bernal and Pinto, in review). Even though lack of midge attraction to silent hosts suggests skin peptides are not involved in host-seeking behavior, it is possible that such chemicals are necessary for host recognition once the midges reach the frog. Further studies that investigate in detail the role of skin peptides in the feeding decisions of frog-biting midges when approaching different frogs are necessary to evaluate the potential role of olfaction in host recognition in this group.

Finally, we found no evidence that frog-biting midges rely on carbon dioxide as a cue to find their host. Although carbon

dioxide alerts many hematophagous insects about a possible opportunity for a blood meal and elicits appetitive searching, the use of this host-emitted cue may be restricted to insects feeding on endothermic hosts. In animals with high metabolic rates, carbon dioxide is a reliable cue of the presence of a host. Humans, for instance, release high quantities of carbon dioxide from the nostrils and very low quantities from their skin (0.3-1.5% of the CO_2 released from the lungs, Tregear 1966). In contrast, in anurans most carbon dioxide is released through their skin (reviewed in Pinder et al. 1992) and given their low metabolic rate, carbon dioxide is excreted in considerably low quantities (Steyermark et al. 2005). Carbon dioxide production has not been measured directly in calling túngara frogs but is estimated to be extremely low (<1.5 ml CO_2 h^{-1}). The low excretion rates of carbon dioxide in anurans, and the rise of the levels of this chemical compound in tropical forests at night (Gillies 1980), probably explain why frog-biting midges do not use carbon dioxide to find their host. Similarly, mosquitoes that feed on ectothermic hosts such as *Culex peccator* and *C. territans* are not attracted to carbon dioxide-baited traps and are thus collected at their resting sites instead (Burkett-Cadena et al. 2013).

Given the low carbon dioxide quantities excreted by anurans, the rates of carbon dioxide used at our traps are unnatural compared to those produced by the host of frog-biting midges. Although the amount of carbon dioxide released by our dry ice and CO_2 tank are effective at attracting several species of mosquitoes, they represent emission rates more than sixty times higher than those estimated by túngara frogs. Such unnaturally high levels of carbon dioxide deterred the frog-biting midges from the traps, even when a mating call was broadcast at the same time. The deterrent effect of carbon dioxide, however, was not present with the lower emission rate of the Z-SeriesTM Capsule Activation (~ 1 ml/min). Even though this device emits a carbon dioxide rate still higher than those estimated for túngara frogs, it is considerably lower than the rates of previous methods used in this study and those produced by devices currently commercially available. We have discussed the possible reasons for why frog-biting midges do not rely on carbon dioxide to find their host but the results here
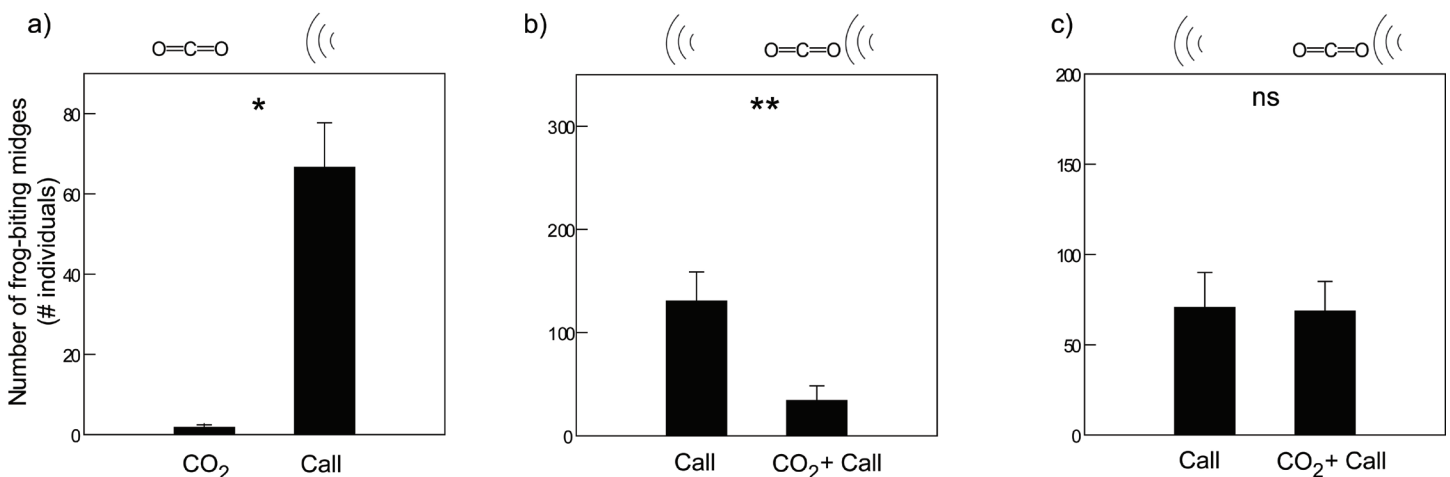

Figure 3. Frog-biting midges attracted to traps emitting carbon dioxide and/or frog calls. Number of midges attracted are shown to: (a) traps releasing carbon dioxide (500 ml/min) vs traps broadcasting mating calls; (b) traps releasing carbon dioxide vs traps broadcasting the mating call and simultaneously releasing carbon dioxide (500 ml/min); (c) traps releasing carbon dioxide vs traps broadcasting the mating call and simultaneously releasing carbon dioxide (1 ml/min). * $p<0.05$, ** $p<0.01$, ns not significant.

also prompt the question, why are frog-biting midges deterred by high carbon dioxide quantities? This behavior could be due to anomalous effects of unnatural carbon dioxide rates, as has been described for other insects (Nicolas and Sillans 1989). It is possible, for instance, that carbon dioxide at such high concentrations acts as an anesthetic, an effect common in insects (Nicolas and Sillans 1989) and also known to occur in frog-biting midges. Although such effects could explain the response to the dry ice experiments, the rates released in our CO_2 tank experiment are equivalent to natural emission rates for mammals and it thus seems unlikely that the deterrent effect of carbon dioxide in those experiments is an artifact of unnaturally high emission rates. It is possible that avoiding plumes of high carbon dioxide concentration allows frog-biting midges to elude large hosts that would be potentially dangerous. Defensive behaviors of the hosts, for instance, can be a significant source of mortality in hematophagous insects (Edman and Scott 1987, Walker and Edman 1985). In general, high cost associated with approaching incorrect hosts are expected to refine the cues used for host-seeking behavior and could explain the decreased response of frog-biting midges to CO_2 rates equivalent to those emitted by animals that are not their hosts. It is currently unclear what mechanism underlies the deterrent effect of high carbon dioxide quantities or whether avoiding such plumes is an adaptation in frog-biting midges. Further studies that investigate the effects of carbon dioxide in the physiology of frog-biting midges and examine the hypothesis that this is an effective strategy to avoid incorrect hosts would need to be formally investigated in the future.

To conclude, host-seeking behavior in frog-biting midges relies on the production of mating calls by its anuran host. Although other cues may be used once they reach their potential victim, mating calls are necessary to elicit long-distance attraction in these biting flies.

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