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Feeding Site Selection by Frog-Biting Midges (Diptera: Corethrellidae) on Anuran Hosts

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Abstract A critical challenge of obtaining a blood meal resides in selecting a feeding site at the host. We investigate the feeding sites of species of frog-biting midges (*Corethrella* spp) on túngara frogs, *Engystomops pustulosus* (Cope), and two species of treefrogs, *Dendropsophus ebraccatus* (Cope) and *Dendropsophus microcephalus* (Cope). To investigate the differences in midge feeding sites, we performed field observations, histological sections of the frogs and examined the mouthparts of the midges. Feeding sites are predicted by the vascular properties of the skin in different body areas of the frogs. According to optimal foraging theory, the midges should feed on host body areas that maximize blood intake. Contrary to optimal foraging theory predictions, however, their feeding sites correspond to body areas with high intensity host defensive behavior.

Keywords Biting behavior · blood feeding · skin characteristics · mouthparts · optimal foraging

Introduction

Given that an organism’s fitness strongly depends on its diet, strong selective pressures refined foraging strategies over evolutionary time. Foraging strategies are thus expected

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to maximize net energy and nutrition intake while minimizing costs involved in acquiring food (MacArthur & Pianka 1966). For haematophagous insects, a critical challenge of obtaining a blood meal resides in locating an appropriate host. Therefore, blood sucking insects have evolved phenotypic traits to optimize host-seeking and feeding behaviors (Balashov 1984). Once haematophagous insects reach their host, however, there is a still another vital step ahead: selecting a biting site. Optimal foraging theory predicts that biting insects should feed on their host on those body areas that maximize blood intake while minimizing costs such as amount of energy to obtain the meal and risk of being injured by their host. Accordingly, hematophagous insects often preferentially feed on specific areas of their host's body. Mosquitoes in the genus *Eretmapodites*, for instance, bite below the knee of a standing human (Haddow 1956) while *Aedes simpsoni* (Theobald) preferentially bite on the head over other body parts of their human host (Haddow, 1946). Feeding site preferences may differ even among closely related mosquito species. *Anopheles gambiae s.s.* (Giles), for example, preferentially bite humans on their feet while the closely related species *Anopheles atroparvus* (Van Theil) prefers to bite humans on the head (De Jong & Knols 1995). Similar to mosquitoes, biting midges are also expected to select feeding sites that maximize the amount of blood they obtain. Accordingly, biting midges that feed on horses, *Culicoides obsoletus* (Meigen) and *C. dewulfi* (Goetghebuer), preferentially land and engorge exclusively at the mane and lower leg regions presumably because they are able to obtain larger blood meals from these areas (Townley et al. 1984).

The feeding sites of temporary ectoparasites are affected by a variety of factors including variation in microclimate, degree of cover and differences in skin thickness on different parts of their host's body (reviewed in Lehane 2005). For example, temperature dependent site selection in the anopluran louse, *Lepidophthirus macrorhini* (Enderlein), restricts their distribution to the hind flippers of their host, elephant seals, *Mirounga leonina* (Linnaeus) (Murray & Nicholls 1965). Degree of cover and skin thickness can also determine the feeding sites of temporary ectoparasites. For example, mosquitoes *Aedes triseriatus* (Say) (Edman & Walker 1985) and different tabanid species, *Tabanus* spp (Mullens & Gerhardt 1979), select different landing and feeding sites on their hosts based on body hair length and density. The ability to obtain a blood meal from different body areas has often been invoked to explain the distribution of biting insects on their host (e.g. Haarløv 1964; Howard et al. 1969; Townley et al. 1984; Walker & Edman 1985; Coleman & Edman 1988). Protection from the host's defensive strategies, however, may also influence ectoparasite distribution on the host. Defensive behaviors of the hosts interfere with mosquitoes' feeding resulting in partial blood meals (Darbro & Harrington 2007) and are a source of significant mortality (Edman & Scott 1987). The lice *Polyplax serrata* (Burmeister), for instance, congregate on the mouse's head and neck where its host cannot reach with its lower incisors when grooming (Murray 1987). In addition, other factors such as cues used to detect their host (Burgess 1959; Kelly 2001), hunger state (Sirinivas et al. 1994) and host's parasites (Chandra & Hati 1993) are known to affect probing and final feeding site selection. Many studies have investigated feeding site selection and distribution on the surface of the host by mosquitoes (Haddow 1956; De Jong & Knols 1995; Knols et al. 1997; Walker & Edman 1985; Dekker et al. 1998; Prior & Torr 2002). In comparison, however, little attention has been given to these behaviors in other blood-sucking flies. In this study we investigate feeding site selection of frog-biting midges, *Corethrella* spp. (Corethrellidae, Diptera), a monogeneric family in which those behaviors have not been previously examined.

Corethrellidae is a family of over 100 species that occurs in tropical and subtropical regions (Borkent 2008; Borkent & Grafe 2012). In this family, female feed on anuran blood cueing on the mating call of frogs to localize a victim and obtain a blood meal (McKeever 1977; Bernal et al. 2006). Although a variety of feeding patterns of frog-biting midges have been anecdotally reported (McKeever 1977; McKeever & French 1991; Bernal et al. 2006; Borkent & Grafe 2012), their feeding site preferences have not been methodologically examined. Early studies report that female *Corethrella* species feed from a variety of areas on the body of frogs and toads. McKeever (1977), for instance, describes that *Corethrella* spp. females seem to preferentially feed on the legs of the bird-voiced treefrog, *Hyla avivoca* (Viosca), in the United States. Similarly, frog-biting midges in North and Central America often feed on the dorsal area and legs of frogs (Bernal *pers. obs*.). In Panama, however, midges attacking túngara frogs (*Engystomops pustulosus* (Cope); formerly known as *Physalaemus pustulosus*) land on the thoracic dorsum of calling males, walk towards the head of the frog and feed at the area around the nostrils (Bernal et al. 2006). Even though the area around the nostrils represents a small proportion of skin available for obtaining a blood meal, the midges aggregate in this area to feed (Fig. 1). Although most biting flies explore with the tip of their labium and probe their host's skin after landing (Walker & Edman 1985; Coleman & Edman 1988; Clements 1999), frog-biting midges rapidly walk directly to the nostrils without stopping suggesting they do not probe until reaching their final feeding site.

In this study we investigated biting site selection by frog-biting midges on their anuran hosts. In particular, we examined the feeding behavior of frog-biting midges attacking túngara frogs by performing field observations, histological sections of the frogs and measuring the mouthparts of the midges. Given that túngara frogs have thick skin with numerous little pustules that give it a toad like appearance (Ibañez et al. 1999), we tested the hypothesis that the characteristics of their skin determine the feeding site selection of the midges. In particular, we address the following questions:

Fig. 1 Frog-biting midges attacking a calling male túngara frog. Engorged female midges are visible at the nostrils of the frog. Photo by Kathrin Lampert

(i) Where do frog-biting midges feed when attacking syntopic frogs of similar size? (ii) Do the properties of the skin of the frogs explain the feeding site selection of the midges? To do so we document site bite selection and skin characteristics of túngara frogs and two similar sized sympatric species of treefrogs (*Dendropsophus ebraccatus* and *D. microcephalus*). Finally, to determine the potential for the midges to bite these frog species on different body areas, we measured the length of the labium across the most common species of midges biting our focal species.

Materials and Methods

Male calling frogs and female frog-biting midges were collected during the rainy season (June–August) at breeding ponds around the facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07.0′N, 79°41.9′W, WGS84 Datum). Field observations of feeding behavior were done by inspecting frogs over five non-consecutive nights when walking around two breeding areas. When engorged midges were found, the location on the body at which it was feeding was marked on a frog silhouette. Those observations were made during the rainy season between 20:00–23:30 h, the hours of highest activity of frog-biting midges and calling behavior of frogs in this area.

To examine skin characteristics of túngara frogs and other anurans of similar size that are also attacked by the midges, we collected one male from each of the following species: túngara frogs (*E. pustulosus*), hourglass treefrogs (*D. ebraccatus*) and yellow cricket treefrog (*D. microcephalus*). These species of treefrogs are similar in size to túngara fogs (*E. pustulosus*: 3.3 cm; *D. ebraccatus*: 2.8 cm, *D. microcephala*: 2.5 cm; Ibañez et al. 1999), and often call from the same breeding areas during the same times. All frogs were brought to the laboratory where they were euthanized using 10 % ethanol following the guidelines jointly published by the North American Herpetological Societies (Guidelines for Use of Live Amphibians and Reptiles in Field Research: Applied Ecology Research Group 1987). For each species, we prepared histological samples of three areas of the thoracic dorsum (anterior, medium and posterior), and the area around the nostrils. All histological samples were fixed in 10 % formalin and processed using standard paraffin-embedding techniques (Luna 1968). Three micrometer Sections three micrometer thick were cut with a rotary microtome across the longitudinal axis of the frogs to obtain nostril sections and along the horizontal axis of the frog to obtain dorsum histological sections. The samples were decalcified using Perenys's method, and then stained using hematoxylin-eosin stains to facilitate microscopic observation (Luna 1968).

We examined the histological sections using a microscope Nikon Eclipse E 200, Japan. We observed a total of 39 microscopic slides containing 117 histological sections distributed as follows: nine slides from the thoracic dorsum of each species of frog (one slide from the anterior, posterior, and middle for each of the three species); and 30 slides from the nostril area, ten from each species. Images were transferred onto a computer screen via Nikon DS camera control unit Ds–L2 with DS camera head. Nine histological sections per slide were studied and digital photos were taken to analyze from each slide. We examined a total of 371 digital images of histological sections from the thoracic dorsum and nostril area. We measured the density of the blood vessels (number of blood vessels per 2 mm^2), shortest distance from the outer surface of the epidermis to the blood vessels (μm) and the surface area of the blood vessels (μm^2). Blood vessels were counted as separate if they were completely independent from adjacent vessels and other dermal cells. All measurements were taken under a real view field 2 mm with a 10× objective, and 10× eyepiece.

Frog-biting midges were captured in the same area where the frogs were collected. The midges were collected using acoustic traps broadcasting the advertisement calls of the frogs. We used modified CDC (Center for Disease Control) miniature light traps, a revised version of the collecting traps used by McKeever and French (1991) following Bernal et al. (2006). Each trap consisted of a Mineroff SME-AFS field speaker placed on the ground with the collecting system directly on top it, and no light source. We used a digital sound pressure level meter (Radio Shack catalogue number 33-2055; C-weighting, fast RMS response) to adjust the amplitude of the call to 82 dB SPL (re. 20μPa) at 1 m, which is equivalent to the sound pressure of a calling túngara frog male at that distance (Ryan 1985). We played a synthetic túngara frog call that was digitally produced by shaping sine waves to the mean values of the parameters of the calls in the population using software developed by J. Schwartz. Details of the parameters used to synthesize the call are described in Ryan (1990). To collect midges attracted to treefrog calls we used natural calls previously recorded in the same area. For each species, we broadcasted 3–5 advertisement calls of ten different individuals to account for within and between individual variations in attractiveness of the mating calls. All stimuli were broadcast using a JVC XL-PG7 CD-player. We used sound traps playing each night the call of only one species of frog for 40 min. We broadcast the mating call of each species for three non-consecutive nights each, for a total of nine nights of trapping. Once closed, the traps were brought to the laboratory where the collecting chambers were refrigerated for at least one hour to euthanize the trapped insects. The specimens were preserved in 75 % alcohol for further analysis. Approximately 600 midges were collected using the sound traps and of those, 500 midges that were in excellent condition were separated for species identification. The midges were identified to species following Borkent (2008) and in this study we focused on the four most common species of midges attacking the frogs.

Although the feeding mechanism of frog-biting midges differs from the piercing mechanism used by mosquitoes, the details of how the midges obtain a blood meal are unclear. Given the lack of an interlocking mechanism, McKeever (1986) proposed frog-biting midges do not snip the skin of their host as Simuliidae (Sutcliffe & McIver 1984) and Ceratopogonidae (McKeever et al. 1995). Accordingly, the presence of finely toothed mandibles and smooth lacinia in frog-biting midges also questions whether they snip the skin of their host to obtain a blood meal (Borkent 2008). Even though it seems unlikely that the midges snip the skin of their host, the mechanisms used by the midges to reach the blood under their host's skin are unknown. Independently of the specific details of the feeding mechanism, the length of the mouthparts constrains how deep into the skin the midges can reach blood vessels. To study the mouthparts of the frog-biting midges, we mounted and prepared 20 microscopic slides with specimens of the four most common species biting the frogs studied here. Specimens in 70 % ethanol were transfer to 2 % KOH solution where they were kept for 2 days. They were then dehydrated by passing them through 70 %, 95 %, and absolute ethanol following Wahid et al. (2003). The head was separated from the body by using a pair of fine insect pins and mounted in Faure's mounting media (Amato José

& Amato 2005). We took photographs (10× objective) of the slides using the same microscope-camera system used to examine the histological sections described above. We measured the length of the labium from the distal point of the gena (μm). This distance is equivalent to the maximum length of the mouthparts involved in feeding and thus provides a conservative measurement of the maximum distance the midges would potentially reach when feeding. To validate our methodology and the use of midges preserved in alcohol for several years, five additional microscopic slides were prepared using *Corethrella appendiculata* (Grabham) and compared to the labium length previously reported for this species by McKeever (1986). Individual *C. appendiculata* midges from the colony maintain in Dr. L. P. Lounibos' Florida Medical Entomology Laboratory at the University of Florida, Vero Beach, were used.

We analyzed the effect of species and body area on the blood vessel distribution, surface area of blood vessels, shortest distance to blood vessels and thickness of the epidermis of the skin histological sections of *E. pustulosus, D. microcephalus* and *D. ebraccatus* performing non-parametric Kruskal-Wallis One-way Analysis of Variance Tests. These analyses were followed by *post hoc* Dwass-Steel-Chritchlow-Fligner Tests (DSCF) for all pairwise comparisons. To further investigate blood vessel characteristics across body areas within each species, we performed Mann–Whitney U Tests. We also examine the length of the proboscis using Two-way Analysis of Variance followed by *post hoc* pairwise comparison using Turkey's HSD post hoc test. All statistical analyses were performed using SYSTAT, version 12. Two-tailed probabilities are given, and the fiducial level of significance, α, was set at 0.05.

Results

Field observations revealed differences in the initial landing sites and feeding sites of frog-biting midges on túngara frogs, hourglass and yellow cricket treefrogs. Midges attacking túngara frogs land on the thoracic dorsum of the males, the region of the body with the largest surface area. Once on their host, they walk towards the head of the frog and feed on the area around the nostrils. Out of 26 túngara male frogs being bitten by the midges in the wild, in 100 % of the cases the midges were feeding at the nostrils (n=38 midges). In a few occasions in the past, in a few occasions frog-biting midges have been seen biting túngara frogs on their legs and by the upper eyelid of the frogs but, as our sample reflects, those cases are unusual. In contrast, midges attacking both species of treefrogs usually land on nearby vegetation and approach the frogs by walking or performing jump-like short flights. Midges bite these species of treefrogs on their legs, thoracic dorsum, and nostrils. In the wild, midges bit the hourglass frogs in the nostrils (66 %) but also frequently feed on their thoracic dorsum (22 %) and legs (12 %) (n=18 midges, 16 male frogs). A similar pattern seems true for the yellow cricket frogs (n=9 midges, 4 nostril, 3 thoracic dorsum, 2 legs, frogs).

Female frog-biting midges from over ten species were captured using sound traps broadcasting mating calls or directly from calling males of the three species of frogs (Table 1). We examined and identify over 500 midges and observed no differences in the most common species of midges that attack and feed from the three species of anurans studied here. In our samples, we observed four species of midges that are highly abundant (*C. bicolor* (Borkent), *C. edwardsi* (Lane), *C. gloma* (Borkent) and *C.*

Table 1 Species of frog-biting midges collected using sound traps broadcasting the mating call of three species of anurans (under "Trap", n=100 specimens per host) and collected manually directly from a frog while they were biting them (under "Frog", n $_{E.\ pustulosus}$=33, n $_{D.\ ebraccatus}$=17, n $_{D.\ microcephalus}$=18)

Species of midges	Host (Species of frog)					
	E. pustulosus		*D. ebraccatus*		*D. microcephalus*	
	Trap	Frog	Trap	Frog	Trap	Frog
C. bicolor Borkent	11	1	1	1	13	0
C. brevivena Borkent	1	0	0	0	0	0
C. carariensis Borkent	0	0	1	0	0	0
C. davisi Shannon	1	0	2	0	0	0
C. douglasi Borkent	1	1	0	1	0	0
C. edwardsi Lane	12	1	26	3	31	0
C. flavitibia Lane	0	1	0	0	0	0
C. globosa Borkent	2	1	0	0	0	0
C. gloma Borkent	20	14	28	5	25	4
C. longituba Belkin	44	13	36	6	21	14
C. manaosensis Lane & Cerqueira	4	1	0	1	1	0
C. quadrivittata Shannon & del Ponte	3	0	6	0	9	0
C. travassosi Lane	1	0	0	0	0	0

Number represent the relative abundance of those species in representative samples of the midges collected using both methods

longituba (Belkin). All of those species were attracted to the call of túngara frogs and the treefrogs as well as found biting frogs from all those species. Túngara frog calls attracted all the species of midges collected in this study while the calls of the treefrogs attracted only a subset of those species (Table 1). Overall, these results suggest that the differences in feeding site selection are not explained by species-specific preferences by the midges attacking each species of frogs.

The skin of túngara frogs is significantly different from that of the treefrogs; results are summarized in Table 2. We examined the characteristics of the skin in the body areas more frequently bitten by the midges: the thoracic dorsum and the area around the nostrils. The vascularization in the thoracic dorsum of túngara frogs is significantly different from the dorsum of treefrogs. Blood capillaries in the thoracic dorsum of túngara frogs are present in lower density than in the treefrogs (KW =13.233, n=93, p=0.0001; Fig. 2a). The density of blood vessels in this body area of túngara frogs is less than half and one quarter of the density in yellow cricket treefrog and hourglass treefrog, respectively. The capillaries at the dorsum of túngara frogs are also smaller (KW =31.728, N=763, p=0.0001; Fig. 2b), the capillaries are about 5.1 % and 6.6 % of the size of the capillaries in treefrogs (túngara frogs: 73.1±22.89 μm^2; yellow cricket treefrog: 1408.4±130.48 μm^2; hourglass treefrog: 1103±93.825 μm^2). The shortest distance, measured from the outer epidermis to each blood vessel, is also significantly different among species at the thoracic dorsum (Table 2). In túngara frogs, the capillaries are at a larger

Table 2 Summary of the statistical tests comparing the characteristics of the skin across species of frogs and their body areas

Skin Characteristics	Grouping variable	Test statistics	*df*	*n*	*p*
Surface area of blood capillaries	Species Dorsum	KW =31.728	2	763	**0.0001**
	Species Nostril	KW=189.63	2		**0.0001**
	Post-hoc pairwise comparison (DSCF test)				
	Ep $_{dorsum}$vs Dm $_{dorsum}$ (p=**0.001**) Ep $_{nostril}$ vs Dm $_{nostril}$ (p=**0.001**)				
	Ep $_{dorsum}$ vs De $_{dorsum}$ (p=**0.001**) Ep $_{nostril}$ vs De $_{nostril}$ (p=**0.001**)				
	Dm $_{dorsum}$vs De $_{dorsum}$ (p=0.254) Dm $_{nostril}$ vs De $_{nostril}$ (p=**0.001**)				
	Dor. Vs Nos. Ep	U=60	1	763	**0.001**
	Dm	U=10,104	1		**0.010**
	De	U=4.039	1		0.952
Shortest distance to blood capillaries	Species Dorsum	KW =205.146	2	763	**0.0001**
	Species Nostril	KW=6.192	2		0.045
	Post-hoc pairwise comparison (DSCF test)				
	Ep $_{dorsum}$Vs Dm $_{dorsum}$ (p=**0.001**) Ep $_{nostril}$ Vs Dm $_{nostril}$ (p=**0.001**)				
	Ep $_{dorsum}$VsDe $_{dorsum}$ (p=**0.0001**) Ep $_{nostril}$ Vs De $_{nostril}$ (p=**0.001**)				
	Dm $_{dorsum}$VsDe $_{dorsum}$ (p=**0.022**) Dm $_{nostril}$ Vs De $_{nostril}$ (p=0.079)				
	Dor. vs Nos. Ep	U=611.0	1	763	**0.0001**
	Dor. & Nos. Dm	U=7,715	1		0.436
	Dor. & Nos. De	U=1352	1		**0.0001**
Density of blood capillaries	Species Dorsum	KW =13.233	2	93	0.001
	Species Nostril	KW=9.071	2		0.011
	Post-hoc pairwise comparison (DSCF test)				
	Ep $_{dorsum}$ Vs Dm $_{dorsum}$ (p=0.001) Ep $_{nostril}$ Vs Dm $_{nostril}$ (p=0.001)				
	Ep $_{dorsum}$ Vs De $_{dorsum}$ (p=0.001) Ep $_{nostril}$ Vs De $_{nostril}$ (p=0.001)				
	Dm $_{dorsum}$ Vs De $_{dorsum}$ (p=0.821) Dm $_{nostril}$ Vs De $_{nostril}$ (p=0.0001)				
	Dor. & Nos. Ep	U=0.050	1	93	0.011
	Dor. & Nos. Dm	U=56	1		0.0001
	Dor. & Nos. De	U=166	1		0.0001
Epidermal thickness	Species Dorsum	KW =26.571	2	763	0.0001
	Species Nostril	KW=51.658	2		0.0001
	Post-hoc pairwise comparison (DSCF test)				
	Ep $_{dorsum}$ Vs Dm $_{dorsum}$ (p=0.441) Ep $_{nostril}$ Vs Dm $_{nostril}$ (p=0.550)				
	Ep $_{dorsum}$ Vs De $_{dorsum}$ (p=0.381) Ep $_{nostril}$ Vs De $_{nostril}$ (p=0.553)				
	Dm $_{dorsum}$ Vs De $_{dorsum}$ (p=0.873) Dm $_{nostril}$ Vs De $_{nostril}$ (p=0.997)				
	Dor. & Nos. Ep	U=521.5	1	763	0.848
	Dor. & Nos. Dm	U=132.5	1		0.000
	Dor. & Nos. De	U=246.5	1		0.000

Kruskal–Wallis Test (KW—for species) or Mann–Whitney test (U—for body areas) are reported for each characteristic of the skin evaluated. *Post hoc* pairwise comparison using Dwass–Steel-Chritchlow–Flinger Test are also shown. Significant results are shown in bold

n Sample size, *df* degrees of freedom, *p* probability, *Ep E. pustulosus* (túngara frog), *Dm D. microcephalus* (Yellow cricket tree frog), *De D. ebraccatus* (Hourglass treefrog)

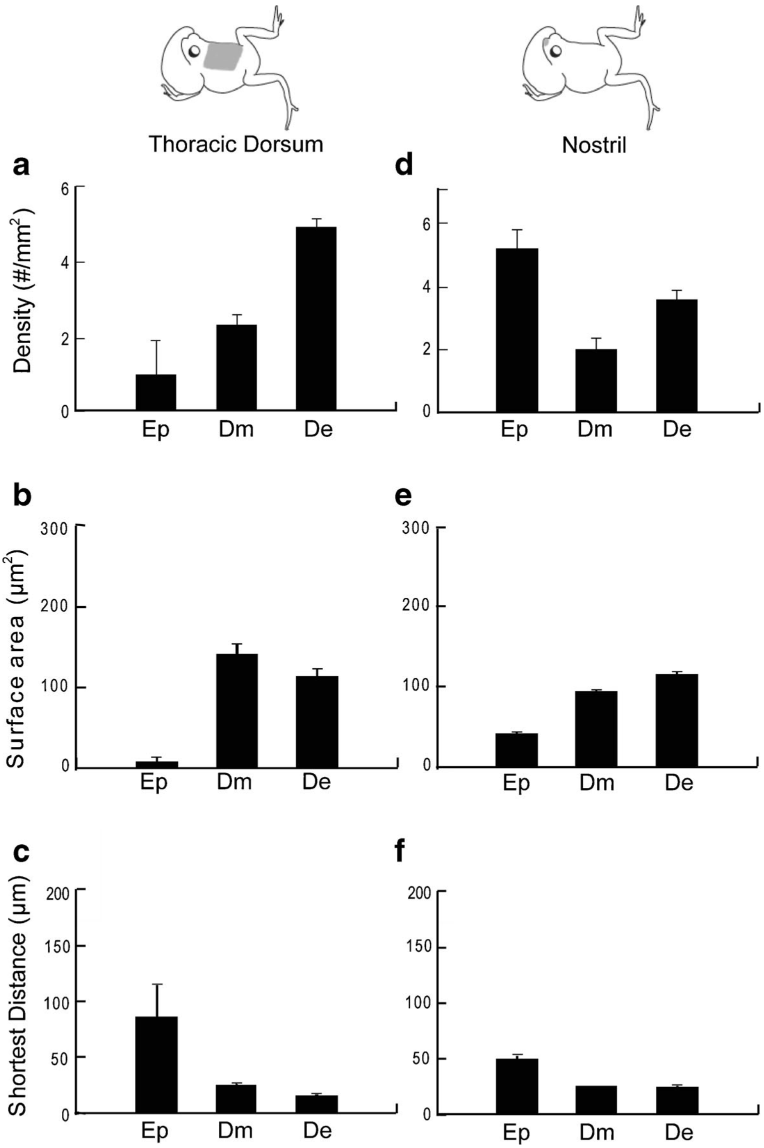
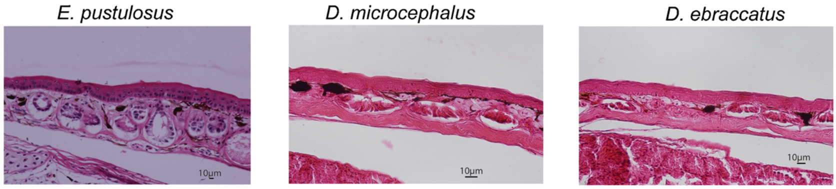


Fig. 2 Vascular characteristics of the skin of túngara frog (Ep, *E. pustulosus*), hourglass treefrog (De, *D. ebraccatus*) and yellow cricket treefrog (Dm, *D. microcephalus*). The characteristics of the capillaries at the thoracic dorsum (**a**–**c**) and nostril (**d**–**f**) of each species are shown

distance from the surface of the skin than those of the treefrogs (KW =205.146, n=763, p=0.001; Fig. 2c). Although the shortest distance to the blood vessels is significantly larger in the skin of túngara frogs than in both of the treefrogs the thickness of the epidermis is not significantly different among species (Table 2). This apparent incongruence is explained by the presence of a thick layer of connective tissue found beneath the epidermis in túngara frogs. Figure 3


a Nostril

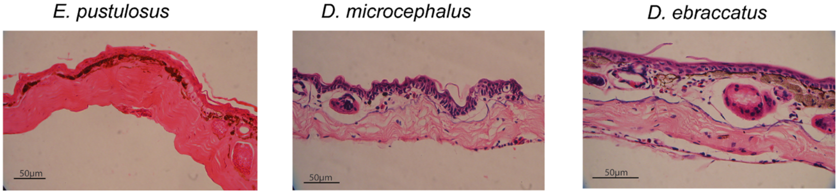

b Thoracic Dorsum

Fig. 3 Sample skin histological sections of túngara frog (*E. pustulosus)* and two sympatric, small treefrogs (*D. microcephalus* and *D. ebraccatus*) at the area of the nostril (**a**) and the thoracic dorsum (**b**)

illustrates this layer in the thoracic dorsum of túngara frogs compared to the skin of the treefrogs in the same body areas.

As expected, the vascularization in the area around the nostrils was significantly different from the thoracic dorsum. Skin sections from the area around the nostrils of túngara frogs are characterized by a significantly higher density of blood vessels than that area in the other species (KW=9.071, $n=93$, $p=0.0001$; Fig. 2d). These blood vessels are also on average of smaller size in túngara frogs than in the treefrogs (KW=189.63; $n=763$, $p=0.0001$; Fig. 2e) but are about at the same distance from the surface compared to capillaries in the treefrogs (KW=6.192, $n=763$, $p=0.045$; Fig. 2f). At the area around the nostrils, the surface area of the blood vessels in túngara frogs is less than half of that on treefrogs.

Our measurements of the length of the labium of *C. appendiculata* confirmed the validity of our methodology. The measurements we obtained are comparable with those reported previously using fresh specimens (McKeever 1986: 194.3–227.14 µm; this study: 190.57–224.24 µm). We thus used the same methodology to examine the length of the labium of the midges from Panama to estimate the distance at which they could potentially access blood vessels below the skin of the frogs. The length of the labium is significantly different between the four most common species of midges ($F_{3,16}=4.549$, $n=20$, $p=0.017$, MSE=60.199), and it varies across a broad range of lengths (170.56–202.78 µm). Only the length of the labium of *C. edwarsi* and *C. gloma*, however, are significantly different (Turkey's Honesty Significant Difference test p=0.017). *Corethrella edwarsi* has the longest labium (197.272 ±4.26 µm) while *C. gloma* has the shortest one (181.5 ±3.84 µm). In *Corethrella quadrivittata* (194.02 ±2.88 µm) and *C. longituba* (184.89 ±2.6 µm) their labium is intermediate in length.

In túngara frogs, the shortest distance from the outer epidermis to the blood vessels is larger at the thoracic dorsum than the nostril ($t=3.165$, $df=114$, $p=0.02$; thoracic dorsum: 67.2–222.46 µm; nostril: 24.33–140.60 µm). On average, the minimum distance from the blood vessels to the surface in the thoracic dorsum of túngara frogs is within the length of the labium of all species of midges. In this body area, however, most capillaries are out of reach given the range of minimum distances to the blood vessel. In contrast, the capillaries in the nostril of this species are all within the range of the length of the labium of the midges. In the treefrog species, the shortest distance to the blood vessels in the thoracic dorsum and nostril are shorter and within the range of length of the labium of the midges (yellow cricket treefrog: thoracic dorsum =6.84–56.84 μm, nostril =5.41–58.1 μm; hourglass treefrog : thoracic dorsum =4.84–34.58 μm, nostril =9.75–127.79 μm).

Discussion

Our results showed differences in the vascular characteristics of the skin of frogs that explain the differences in feeding site preferences of frog-biting midges among host species. While several studies have shown that some species of blood-sucking insects feed on body areas of their host that potentially increase their ability to obtain a blood meal (reviewed in Lehane 2005), to our knowledge this is the first study to directly examine the vascular properties of the host's skin to test the hypothesis that the distribution and abundance of blood vessels determines their feeding preferences. Frog-biting midges attacking túngara frogs do not feed from the thoracic dorsum of the frog where there are few, small capillaries located deep in the skin below a thick layer of connective tissue. At the area around the nostrils, the preferred biting site of the midges, blood vessels are larger and more abundant than at the thoracic dorsum. In the treefrogs, however, the contrast between the abundance and location of the blood vessels between the thoracic dorsum and the area around the nostrils is minor in comparison. The difference between the features of the skin of túngara frogs and the treefrogs suggests the feeding decisions of the midges are explained by the skin characteristics of their host.

In túngara frogs, the vascular characteristics of the skin at the area around the nostrils facilitate obtaining a blood meal by the midges. An important aspect to consider, however, is how those properties of the skin compare to the mouthparts of the midges. There is evidence for close correlations between the size and shape of the mouthparts and the feeding habits of blood-sucking insects (reviewed in Lehane 2005). For example, several species of tabanids (*Chrysops vittatus* (Wiedemann), *Hybomitra lasiophthalma* (Macquart) and *Tabanus lineola* (Fabricius)) with longer proboscis feed on animals with deeper or thicker hair (Lall & Davies 1971; Mullens & Gerhardt 1979). The biting mechanism of frog-biting midges has not been described yet. The length of the labium, however, provides a conservative estimate of the distance the midges can reach into the skin to obtain blood. Although we found variation in the length of the labium among midge species, our results are consistent with the hypothesis that distribution and size of the blood vessels determines the body areas where the midges feed. In the species of frog-biting midges feeding from the anurans in this study, the length of the labium is long enough to reach the capillaries at the area around the nostrils in túngara frogs but they have limited access to the capillaries at the thoracic dorsum of this frog species. In contrast, given the length of their labium, the midges are able to obtain a blood meal from both the thoracic dorsum and the area around the nostrils of treefrogs. The depth of the capillaries at the thoracic dorsum in túngara frogs combined with their low density, small size and the thick layer of connective tissue separating them from the surface, restrict the opportunities for the midges to feed from this body area. Based on our findings, we propose that the selective pressures that promoted vascular characteristics at the area around the nostrils for efficient gas exchange, also provide an opportunity for frog-biting midges to efficiently obtain a blood meal at those areas.

Several studies investigating feeding site selection support the hypothesis that easiness to obtain a blood meal determines biting site selection in heamatophagous insects (reviewed in Lehane, 2005). For example, *Aedes iriserialus* (Hasselquist) limit their feeding sites to areas where their host's hair is short and sparse such as the ears, eyelids, nose and feet (Walker & Edman 1985). Easiness to obtain a blood meal has been repeatedly invoke as an explanation for feeding site preferences and this behavior has been attributed to the use of cues such as variation in skin temperature, moisture or color, and body odor of the host, among others (De Jong & Knols 1995; Barrozo & Lazzari 2004; Khan & Maibach 1966; Bowen 1991; Wang et al. 2009). In most blood feeding mosquitoes (Culicidae) and biting midges (Ceratopogonidae) studied to date, probing and feeding site selection are based on thermal gradients, carbon dioxide (CO_2) and moisture emanating from the host (Hocking 1971). Given the widespread role of CO_2 as an important cue for host location by blood-sucking insects (Gillies 1980; Nicolas & Sillans 1989) and the lack of temperature differences across the body of túngara frogs (Bernal *unpub. data*), CO_2 could act as a cue used in feeding site selection by frog-biting midges. It is possible that the midges bite túngara frogs in the area around the nostrils following higher CO_2 concentrations emanating from them. The use of CO_2 by frog-biting midges in feeding site selection once they reach their host, however, is improbable. Although in anurans gas exchange takes places at the nostrils, frogs excrete most CO_2 through the skin (Boutilier et al. 1992; Wells 2007). In addition, frog-biting midges also feed at the thoracic dorsum and legs of treefrogs suggesting that, if involved, CO_2 is not the main driver of feeding site selection. The nostrils are also in close proximity to the vocal sac, where most of the sound of the mating call emanates from the frog. The midges could thus be following a sound intensity gradient that leads them to the nostrils. The use of sound to choose a feeding site at their host is, however, also questioned by the midges biting the treefrogs in areas different from the nostrils. Although further research that directly tests the use of CO_2 and sound as cues to select a feeding site is necessary, our results suggest those factors, if involved, play a minor role in feeding site selection in frog-biting midges.

In general, biting insects prefer feeding sites where they are less likely to suffer from the defensive behavior of their host (Edman & Kale 1971; Edman & Walker 1985; Davies 1990; Darbro & Harrington 2007). It is thus unexpected that frog-biting midges feed preferentially at the area around the nostrils where túngara frogs frequently swat them using their front feet (Bernal *pers. obs.*). Previous studies have shown that mosquito feeding success is reduced on vertebrate hosts that perform more defensive behaviors (Blackmore & Dow 1958; Edman & Kale 1971; Webber & Edman 1972; Klowden & Lea 1978; Walker & Edman 1985; Cully et al. 1991). In addition, movement and slapping by the host considerably increases the risk of obtaining a blood meal by a biting fly. In several species of mosquitoes, for instance, host defensive behavior discourages them from feeding and is a significant source of insect mortality (Edman & Scott 1987; Darbro & Harrington 2007; Kweka et al. 2010). Host defensive behavior is considered a significant driver promoting rapid blood feeding in body areas of the host that maximize nutrient uptake rates while reducing injuries or mortality (Gillett 1967). Tabanids, for example, preferentially feed from the legs and back of cattle and the mane, lower leg and hind quarters of a horse where their hosts defensive behavior are less efficient (Magnarelli & Anderson 1980; Townley et al. 1984; Braverman 1988; Dekker et al. 1998). In the case of frog-biting midges, their anuran hosts are also known to repeatedly move their legs attempting to dislodge attacking midges (McKeever 1977; Bernal et al. 2006). In our study we found that frog-biting midges feed from túngara frogs in the area of the body with the highest defensive behaviors by their host. Although túngara frog males are calling when they are approached by midges, they often move their front feet attempting to dislodge them. A male túngara frog can produce up to 153 swats with their front feet in 195 s when attacked by midges in the wild (44.76±33.97 swats during 50 mating calls, Bernal *unpublished data*). By feeding at the nostrils, however, the midges maximize their blood intake given the abundant, larger capillaries in this area. Our findings suggest that the characteristics of the skin at the thoracic dorsum prevent the midges from feeding in that area even though it may come at a cost imposed by the frog's defensive behaviors. Further studies that investigate the feeding success and mortality of the midges in relation to the defensive strategies of túngara frogs are necessary to improve our understanding of the trade-offs confronted by these midges.

Our results show that the differences in feeding sites on túngara frogs and the treefrogs are not due to different species of midges having different feeding strategies. Midges from all species were attracted to the calls of the three species of frogs and/or directly collected biting them. Given the distinctive feeding patterns when the midges obtain a blood meal from túngara frogs versus the two treefrogs species, and the fact that the same species of midges attack these three species of frogs, we are confident that the difference in feeding sites are not determined by differences among species of midges. We are currently performing studies designed to investigate the specificity of the relationships between frog-biting midges and their hosts to find conclusive answers about the degree of specificity between these eavesdroppers and their victims.

To our knowledge, no previous studies have investigated the vascular properties of the skin to explain feeding site preferences of blood-sucking insects. In addition, our study is the first demonstration that frog-biting midges attacking túngara frogs preferentially feed at the nostrils where the vascular characteristics of the skin favor obtaining a blood meal. Our findings emphasize the tradeoffs confronted by biting insects and describe a feeding strategy that may pay high costs imposed by the defensive behaviors of the host. Ultimately this study highlights the importance of investigating feeding site selection incorporating the vascular properties of the skin to obtain a comprehensive understanding of the tradeoffs of this behavior.

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References

Amato José FR, Amato SB (2005) New species of *Temnocephala Blanchard* (Platyhelminthes, Temnocephalida) ectosymbiont on giant water bugs, Belostoma spp. (Hemiptera, Belostomatidae) from southern Brazil. Rev Bras Zool 22:107–118

Balashov YS (1984) Interation between blood-sucking arthropods and their hosts, and its influence on vector potential. Annu Rev Entomol 29:137–156

Barrozo RB, Lazzari CR (2004) The response of the blood-sucking bug *Triatoma infestans* to carbondioxide and other host odours. Chem Senses 29:319–329

Bernal XE, Rand AS, Ryan MJ (2006) Acoustic preferences and localization performance of blood-sucking flies (*Corethrella* Coquillett) to túngara frog calls. Behav Ecol 17:709–715

Blackmore JS, Dow RP (1958) Differential feeding of *Culex tarsalis* on nestling and adult birds. Mosq News 18:15–17

Borkent A (2008) The frog-biting midges of the world (Corethrellidae: Diptera). Zootaxa 1804:5–350

Borkent A, Grafe TU (2012) The frog-biting midges of Borneo-from two to eleven species (Corethrellidae: Diptera). Zootaxa:1–45

Boutilier RG, Stiffler DF, Toews DP (1992) Exchange of gases, ions and water in aquatic amphibians. In: Feder ME, Burggren WW (eds) Environmental physiology of the amphibians. University of Chicago Press, Chicago

Bowen MF (1991) The sensory physiology of host seeking behavior in mosquitoes. In: Mittler, T. E., F. J. Radovsky and V. H. Resh:139–158

Braverman Y (1988) Preffered landing sites of culicoides species (Diptera, Ceratopogonidae) on a horse in Israel and its relevance to summer seasonal recurrent dermatitis (sweet itch). Equine Vet J 20:426–429

Burgess L (1959) Probing behaviour of *Aedes aegypti* (L.) in response to heat and moisture. Nature 184:1968–1969

Chandra G, Hati AK (1993) Correlation between the preferred biting site of *Culex quinquefasciatus* and the region of the body affected by clinical filariasis. Ann Trop Med Parasitol 87:393–397

Clements AN (1999) The biology of mosquitoes; Sensory reception and behavior. CABI Publishing Wallingford, Oxon

Coleman RE, Edman JD (1988) Feeding-site selection of *Lutzomyia longipalpis* (Diptera: Psychodidae) on mice infected with *Leishmania mexicana amazonensis*. J Med Entomol 25:229–233

Cully J, Heard P, Wesson D, Craig G (1991) Antibodies to La Crosse virus in eastern chipmunks in Indiana near an *Aedes albopictus* population. J Am Mosq Control Assoc 7:651–653

Darbro JM, Harrington LH (2007) Avian defensive behavior and blood-feeding success of the West Nile vector mosquito *Culex pipiens*. Behav Ecol 18:750–757

Davies CR (1990) Interrupted feeding of blood-sucking insects: causes and effects. Parasitol Today 6:19–22

De Jong R, Knols BGJ (1995) Selection of biting sites on man by two malaria mosquito species. Experientia 51:89–99

Dekker T, Takken W, Knols BGJ, Bouman E, Van de Laak S, de Bever A, Huisman PWT (1998) Selection of biting sites on a human host by *Anopheles gambiae* s.s., *An. arabiensis* and *An. quadriannulatus*. Entomol Exp Appl 87:295–300

Edman J, Kale H (1971) Host behavior: its influence on the feeding success of mosquitoes. Ann Entomol Soc Am 64:513–516

Edman JD, Scott TW (1987) Host defensive behavior and the feeding success of mosquitoes. Insect Sci Appl 8:617–622

Edman JD, Walker JE (1985) Vector-host interplay-factors affecting disease transmission. In: Lounibos LP, Ray JR, Frank JH (eds) Ecology of mosquitoes. Chapman & Hall, New York, pp 273–285

Gillett JD (1967) Natural selection and feeding speed in a blood-sucking insect. Proc R Soc B 167:316–329

Gillies MT (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae). Bull Entomol Res 70:525–532

Haarløv N (1964) Life cycle and distribution pattern of Lipoptenacervi (L.)(Dipt., Hippobosc.) on Danish Deer. Oikos 15:93–120

Haddow AJ (1946) The mosquitoes of Bwamba County. Uganda Bull Entomol Res 37:57–82

Haddow AJ (1956) Observations on the biting-habits of African Mosquitos in the Genus *Eretmapodites* Theobald. Bull Entomol Res 46:761–772

Hocking B (1971) Blood-sucking behavior of terrestrial arthropods. Annu Rev Entomol 16:1–26

Howard MMD, Khan AA, Walter G, Strauss MD, Skinner WA (1969) Human skin in relationship to mosquito attraction and repulsion. Conn Med 33:23–28

Ibañez R, Rand AS, Jaramillo CA (1999) Los anfibios del Monumento Natural Barro Colorado, ParqueNacionalSoberanía y areas adyacentes. Mizrachi. E and SA Pujol, Santa Fe de Bogota

Kelly DW (2001) Why are some people bitten more than others? Trends Parasitol 17:578–581

Khan AA, Maibach HI (1966) Quantitation of effect of several stimuli on landing and probing by *Aedes aegypti* (L).J. Econ Entomol 59:902–905

Klowden M, Lea A (1978) Blood meal size as a factor affecting continued host-seeking in *Aedes aegypti* (L.). Am J Trop Med Hyg 27:827–831

Knols BGJ, Takken W, Cork A, De Jong R (1997) Odour-mediated host-seeking behaviour of *Anopheles* mosquitoes: a new approach. Ann Trop Med Parasitol 91:117–118

Kweka E, Mwang'onde B, Lyaruu L, Tenu F, Mahande A (2010) Effect of different hosts on feeding patterns and mortality of mosquitoes (Diptera: Culicidae) and their implications on parasite transmission. J Glob Infect Dis 2:121–123

Lall SB, Davies DM (1971) An intergeneric comparison of cephalic structure in Tabanids (Diptera) in relation to feeding habits. J Med Entomol 8:700–706

Lehane M (2005) The biology of blood sucking insects. Cambridge University Press, London

Luna LG (1968) Manual of histological staining methods of the Armed Forces Institute of Pathology. Mc Graw Hili, New York, pp 9–20

MacArthur RH, Pianka ER (1966) On the optimal use of a patchy environment. Am Nat 100:603–609

Magnarelli LA, Anderson JF (1980) Feeding behavior of Tabanidae (Diptera) on cattle and serologic analyses of partial blood meal. Environ Entomol:664–667

McKeever S (1977) Observations of *Corethrella* feeding on tree frogs (Hyla) Mosq. News 37:522–523

McKeever S (1986) Mouthparts of the four North American Corethrella species (Diptera: Chaoboridae), with Detailed study of *C. appendiculata*. J Med Entomol 23:502–512

McKeever S, French FE (1991) Corethrella (Diptera, Corethrellidae) of eastern North America:laboratory life history and field responses to anuran calls. Ann Entomol Soc Am 84:493–497

McKeever S, Hagan DV, Grogan WL (1995) Comparative study of mothparts of predaceous midges of the tribe Palpomyiini (Diptera, Ceratopogonidae) from eastern-United-States. Proc Entomol Soc 97:799–832

Mullens BA, Gerhardt RR (1979) Feeding behavior of some Tennessee Tabanidae. Environ Entomol 8:1047–1051

Murray MD (1987) Effects of host grooming on louse populations. Parasitol Today 3:276–278

Murray M, Nicholls D (1965) Studies on the ectoparasites of seals and penguins. in The ecology of the louse *Lepidophthirus macrorhini* Enderlein on the southern elephant seal, *Mirounga leonina* (L). Aust J Zool 13:437–454

Nicolas G, Sillans D (1989) Immediate and latent effects of carbon dioxide on insects. Annu Rev Entomol 34: 97–116

Prior A, Torr S (2002) Host selection by *Anopheles arabiensis* and *An. quadriannulatus* feeding on cattle in Zimbabwe. Med Vet Entomol 16:207–213

Ryan MJ (1985) The túngara frog, a study in sexual selection and communication. University of Chicago Press, Chicago

Ryan MJ, Rand AS (1990) The sensory basis of sexual selection for complex calls in the túngara frog, *Physalaemus pustulosus* Behav. Ecol. Sociobiol. (Sexual selection for sensory exploitation). Evolution 44:305–314

Sirinivas SD, Selvaraj RP, Dwarakanath SK (1994) Biting behavior of *Armigeres subalbatus* (Coquillett) with reference to host selection and landing. Indian J Exp Biol 32:348–350

Sutcliffe JF, McIver SB (1984) Mechanics of blood-feeding in black flies (Diptera, simuliidae). J Morphol 180:125–144

Townley P, Baker KP, Quinn J (1984) Preferential landing and engorging sites of Culicoides species landing on a horse in Ireland. Equine Vet J 16:117–120

Wahid I, Sunahara T, Mogi M (2003) Maxillae and mandibles of male mosquitoes and female autogenous mosquitoes (Diptera: Culicidae). J Med Entomol 40:150–158

Walker ED, Edman JD (1985) Feeding-site selection and blood-feeding behavior of *Aedes triseriatus* (Diptera, Culicidae) on rodent (Sciuridae) hosts. J Med Entomol 22:287–294

Wang G, Qiu Y, Lu T, Kwon H, Pitts R, Van Loon J, Takken W, Zwiebel L (2009) *Anopheles gambiae* TRPA1 is a heat-activated channel expressed in thermosensitive sensilla of female antennae. Eur J Neurosci 30: 967–974

Webber LA, Edman JD (1972) Anti-mosquito behaviour of ciconiiform birds. Anim Behav 20:228–232

Wells KD (2007) The ecology and behavior of amphibians. University of Chicago Press, Chicago