

Molecular Confirmation of Frogs (Anura) as Hosts of Corethrellidae (Diptera) in the Southeastern United States

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Abstract

Flies in the family Corethrellidae Edwards 1932 (Diptera) are known to be attracted to the mating calls of male frogs. For the first time, the hosts of corethrellids were identified to species by analyzing bloodmeals taken from resting female flies. A portion of the cytochrome *b* gene was amplified and sequenced from blood-engorged flies using vertebrate-specific primers. The flies were collected over 6 yr at two locations in the southeastern United States from resting boxes and natural resting sites (rodent burrows). Potential host abundance focused on frog surveillance, and estimation relied on visual encounters, passive trapping (artificial refugia), and call surveys. This study confirms that corethrellids take blood from tree frogs (Hylidae); however, it was found that true frogs (*Lithobates* Fitzinger 1843 (Ranidae: Anura) sp.) were the principal host selected by *Corethrella brakeleyi* (Coquillett 1902) (~73% of identified bloodmeals). These preliminary data suggest that host selection of *Corethrella* Freeman 1962 sp. is not necessarily correlated with host calling abundance.

Key words: frog, amphibian, bloodmeal, hematophagy, *Lithobates sphenoccephala*

Members of the family Corethrellidae (Diptera) have been observed taking bloodmeals from frogs and are known to be attracted to the mating calls of male frogs (McKeever 1977, Bernal et al. 2006). The most attractive call for female Nearctic corethrellids was determined in field tests to be the call of male *Hyla gratiosa* LeConte 1856 (Hylidae: Anura) (McKeever and French 1991). In the neotropics, field and laboratory tests using *Engystomops pustulosus* Cope 1864 (Leptodactylidae: Anura) calls revealed that increased calling rate and increased call complexity are the most attractive qualities of the acoustic signal to female corethrellids (Bernal et al. 2006, de Silva et al. 2015). In this case, these are also traits that are more attractive to female frogs. The flies are thus evaluating an honest mating signal, and their eavesdropping behavior may affect host fitness (e.g., blood loss or as vectors of trypanosomes) (Johnson et al. 1993, Camp 2006, de Silva et al. 2015, Meuche et al. 2016). Currently, it is unknown if the acoustic preference of the flies reflects actual feeding patterns in nature, or if the flies are feeding opportunistically.

In determining the natural vertebrate hosts of any group of hematophagous insects, the method that provides the least biased evidence is the identification of host blood in the midgut of the insects collected from a natural resting habitat. This avoids the sampling bias of using sentinel animals, and assumes that engorged resting female flies prefer a similar habitat regardless of host. The first identification of the host from a blood fed corethrellid suggested that mammals and birds may also be hosts (Williams and Edman 1968). To date, no other identification of hosts using bloodmeals has been published; however,

corethrellids around the world may be observed feeding on calling male frogs (McKeever 1977, Bernal et al. 2006, Borkent and Belton 2006, Borkent and Grafe 2012). Herein we provide the first analysis of the hosts of corethrellids using ingested blood in female flies collected from two sites in the southeastern United States over 6 yr. Information about host abundance was recorded at each site to determine if corethrellids are feeding on frogs as expected, and to provide preliminary data to test the hypothesis that corethrellids are feeding opportunistically.

Materials and Methods

Survey of Corethrellid Population

In 2001–2004, corethrellids were collected from a site in Tuskegee National Forest (TNF), Macon County, Alabama (32°25.94420'N 85°38.676'W). At TNF, black wooden resting boxes and natural resting sites (rodent burrows) were sampled twice a week in the morning with a backpack aspirator (Cupp et al. 2004). In 2005 and 2006, corethrellids were collected from bird pond (BP) near Statesboro, Georgia (32°23.8'N 81°46.4'W) from resting boxes modified by removing the bottom panel. At BP, nylon stockings were used on the collection cups to prevent loss of flies through the wire mesh. Insects were anesthetized with CO₂ gas or by freezing at –20°C for 1 min and sorted to species (Stone 1968) on an ice-cold metal plate. Damaged specimens are listed as ‘C. sp.’ (Table 1) due to difficulties in observing and counting flagellar sensoria with a binocular microscope (Stone 1968). Individual blood-fed female corethrellids were placed into microcentrifuge tubes

Table 1. Resting box collections of *Corethrella* spp. over 6 yr at two different locations (Tuskegee National Forest, Alabama = 'TNF', Bird Pond, Statesboro, Georgia = 'BP'), including the number of blood-fed females (BM) collected

Site	Year	<i>C. brakeleyi</i> (BM)	<i>C. wirthi</i> (BM)	<i>C. sp.</i> (BM)	Total (BM)
TNF	2001	35 (2)	2 (2)	8 (2)	45 (6)
TNF	2002	45 (5)	2 (2)	42 (0)	89 (7)
TNF	2003	90 (7)	2 (2)	19 (0)	111 (9)
TNF	2004	2 (2)	0	0	2 (2)
BP	2005	39 (26)	8 (5)	0	47 (31)
BP	2006	54 (15)	8 (3)	0	62 (18)
Total		265 (57)	22 (14)	69 (2)	356 (73)

and stored continuously at -70°C until processing, which took place in 2006. All collections were performed between March and October.

Survey of Anuran Population

Visual encounters of anurans were recorded throughout the season at TNF and BP. Tree frogs (Hylidae) were expected to be the main host (McKeever and French 1991); therefore, 15 artificial tree frog refugia constructed out of polyvinyl choride pipes were positioned 2 m from the ground on trees near the resting boxes at the BP site (Moulton et al. 1996, Boughton et al. 2000). Calling surveys of male frogs were conducted on nights prior to resting box sampling at BP. The relative number and species of calling frogs were noted for the BP site three times per night beginning at sunset. The calling male frog survey data combined with refugia and visual encounters were used to generate a qualitative rank of each species at BP based on encounter frequency over the course of the study. The ranked calling scores were compared to feeding rate at Bird Pond using Spearman's *rho* non-parametric rank correlation test.

Bloodmeal Identification

Field-collected corethrellid flies were homogenized and DNA was extracted and purified using a commercial kit (DNEasy; Qiagen Inc., Germantown, MD). A polymerase chain reaction was performed using *Taq* polymerase and the following primers, which were designed to amplify a segment of Nearctic reptile and amphibian cytochrome B: 5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3' and 5'-GCH GAY ACH WVH HYH GCH TTY TCH TC-3' (H=A, C, or T, Y=C or T, and V=A, C, or G.) (Cupp et al. 2004). The products were analyzed by agarose gel electrophoresis and amplicons were sequenced (ABI 3730XL, Applied Biosystems, Inc., Foster City, CA). Hosts were identified using the basic local alignment search tool (BLAST) available from the National Center for Biotechnology and Information (National Institutes of Health, www.ncbi.nlm.nih.gov). Additionally, an alignment was made with representative native and non-native anurans and the sequences taken from corethrellid bloodmeals using the Muscle algorithm in MEGA software, version 6.0 (Tamura et al. 2013). A maximum likelihood tree was made to visualize genetic similarity (Kimura 2-parameter method) and to provide additional support for the putative identifications (Supplemental Figure 1).

Results

In total, 356 female corethrellids were collected from resting boxes or natural sites (Table 1). *Corethrella brakeleyi* was the most abundant species captured at each location (68.8% of the total at TNF; 85.3% of the total at BP). There were a total of 57 blood-fed female *C. brakeleyi* sampled, and 14 blood-fed *Corethrella wirthi* Stone 1968 collected from both BP and TNF (Table 1). The hosts were identified from 19 bloodmeals from *C. brakeleyi* taken from both sites over the course of the study (seven (31.8%) from TNF and the remaining from BP)

Table 2. Hosts of *Corethrella* spp. based on bloodmeal analysis

Site	Host species	<i>C. brakeleyi</i>	<i>C. wirthi</i>
TNF ^a	<i>D. avivoca</i>	1	2
	<i>D. chrysosecelis</i>	0	1
	<i>L. clamitans</i>	1	0
	<i>L. sphenoccephala</i>	2	0
BP ^b	<i>A. gryllus</i>	2	0
	<i>Lithobates clamitans</i>	5	0
	<i>L. sphenoccephala</i>	8	0

^aTuskegee National Forest, Alabama.

^bBird Pond near Statesboro, Georgia.

(Table 2). Of the hosts of *C. brakeleyi*, most were identified to be *Lithobates* sp. ($n = 16$, 72.7%), with *Lithobates sphenoccephala* (Cope 1886) being fed upon at both sites and comprising nearly half of the species identified ($n = 10$, 45.4%) (Table 2). The hosts of three *C. wirthi* species were identified as tree frogs (Hylidae) at TNF: *Dryophytes avivoca* Driosa 1928 (Hylidae: Anura) ($n = 2$) and *D. chrysosecelis* Cope 1880 (Hylidae: Anura) ($n = 1$) (Table 2). Bloodmeals were also taken from bronze frog, *Lithobates clamitans* Latreille 1801 (Ranidae: Anura), at both sites ($n = 6$). Cricket frogs ($n = 2$, *Acris gryllus* LeConte 1825 (Hylidae: Anura)) were fed upon by *C. brakeleyi* at BP but not at TNF. Host identifications were from May through September, and there was no obvious seasonality to the collections. Due to the low number of identifications (28.8% of total bloodmeals), optimizations to the PCR assay were performed with no success. Matching sequence identity to published databases produced good matches, and sequence alignment confirmed these host identifications (Suppl Fig. 1 [online only]).

The TNF site had 12 anuran species, and the species composition agrees with published data (Mount 1980) (Table 3). Cricket frogs (*A. gryllus* sp.) were frequently heard and encountered at TNF, which agrees with other published amphibian surveys from this site (Burkett-Cadena et al. 2008), as well as large choruses of *D. avivoca* and *D. chrysosecelis*. The BP site had 11 anuran species (Table 3), of which, the green tree frog (*Dryophytes cinerea* Schneider 1799 (Hylidae: Anura)) was the most abundant frog. Other frogs in large choruses at the BP site were *A. gryllus* and *Gastrophryne carolinensis* Holbrook 1835 (Microhylidae: Anura) (Table 3). True frogs (*Lithobates* sp.) were often seen along the shoreline and around small puddles at BP, and infrequently heard calling in groups of five or less. There was no correlation between host rank abundance and feeding rate at BP (Spearman's $\rho = 0.22$, $df = 11$, $P > 0.05$).

Discussion

For the first time, the identification of host species from corethrellid bloodmeals is reported. All hosts were identified to be from frogs, as expected (McKeever 1977). The hosts identified from TNF (4 of 7, 57.1%) were commonly heard anurans (*D. avivoca* and *D. chrysosecelis*),

Table 3. Anuran species abundance at two sites where blood-fed *Corethrella* spp. were analyzed for their host selection

Species	TNF	BP	Rank
<i>Lithobates catesbeiana</i> (Shaw 1802)	Present	Present	3
<i>Lithobates clamitans</i>	Present	Present	8
<i>Lithobates gryllio</i> (Stejneger, 1901)	Absent	Present	5
<i>L. sphenocéphala</i>	Present	Present	10
<i>D. avivoca</i>	Present	Absent	–
<i>D. cinerea</i>	Present	Present	1
<i>D. chrysoscelis</i>	Present	Absent	–
<i>Dryophytes femoralis</i> (Daudin 1800)	Present	Present	7
<i>A. gryllus</i>	Present	Present	2
<i>Pseudacris crucifer</i> (Wied-Neuwied 1838)	Present	Present	10
<i>Pseudacris nigrita</i> (LeConte 1825)	Present	Present	10
<i>Anaxyrus fowleri</i> (Hinckley 1882)	Present	Absent	–
<i>Anaxyrus terrestris</i> (Bonnaterre 1789)	Absent	Present	6
<i>G. carolinensis</i>	Present	Present	4

Anuran species are listed as present/absent based on visual encounter surveys and passive trapping (refugia) at Tuskegee national Forest (TNF), Alabama, and Bird Pond (BP) in Statesboro, Georgia. Calling surveys were conducted semi-regularly from May to August at BP, and the species were ranked (with ties) based on visual encounters, size of the chorus, and frequency of the calling (e.g., *D. cinerea* was considered to be the most abundant frog).

whereas the majority (13 of 15, 86.7%) of hosts at BP were identified as *L. clamitans* or *L. sphenocéphala* – both were seldom heard in calling surveys; however, these frogs were frequently encountered visually. Thus, *C. brakeleyi* may be using other cues to locate hosts (Bernal and de Silva 2015), or frogs may be balancing the costs of parasitism with benefits of attracting a mate by calling more infrequently in areas of intense parasitism (Bernal et al. 2007). Corethrellids in other biogeographic regions are attracted to the calls of Nearctic tree frogs, as well as local frog species (Bernal et al. 2006, Borkent and Belton 2006, Borkent and Grafe 2012), supporting the hypothesis that host specificity is low in these midges, and the midges are feeding opportunistically on calling hosts. Others have noticed a correlation between relative attractiveness of the calls of frog species to corethrellids and the prevalence of trypanosomes vectored by corethrellids in the male frog population (Meuche et al. 2016), supporting the hypothesis that the flies are using sound as a primary cue to locate hosts. Many studies have focused on the specific features of attractive calls (McKeever and French 1991, Bernal et al. 2006, Camp 2006, Borkent and Grafe 2012, Bernal and de Silva 2015, de Silva et al. 2015). To date, *Lithobates* spp. were not assumed to be hosts of corethrellids in the Nearctic, and the relative attractiveness of their respective calls has not been determined. The data presented here illustrate that more studies should be performed to understand the relationship between the host attraction and host selection in this group of midges.

While PCR-based molecular diagnostic assays are a superior method for identifying hosts from the guts of hematophagous insects, successful identification may depend on predicting the target hosts (Kent 2009). The PCR assay used here has been shown to be useful for the identification of reptile and amphibian bloodmeals (Cupp et al. 2004) as well as for birds and mammals (Patrican et al. 2007); however, hosts were identified from <30% of the sampled bloodmeals. Similar studies on host feeding patterns have failed to conclusively identify host blood from some mosquito species that are suspected of feeding solely on ectothermic vertebrates (e.g., *Uranotaenia sapphirina* Osten Sacken 1868 (Culicidae: Diptera)) (Irby and Apperson 1988, Cupp et al. 2004, Burkett-Cadena et al. 2008). Therefore, the existence of additional hosts cannot be ruled out.

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