Host Attraction and Host Selection in the Family Corethrellidae (Wood And Borkent) (Diptera)

Jeremy Vann Camp
Georgia Southern University

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Flies in the family Corethrellidae (Diptera) are known to be attracted to the mating calls of tree frogs. Field studies using the calls of nine species of frogs native to the SE USA confirmed prior findings that the call of the barking tree frog, *Hyla gratiosa* LeConte, is the most attractive call to sympatric *Corethrella* (Coquillett) from Georgia. A synthetic barking tree frog call was created using computer software. It was found that the synthetic call was more attractive than the real call of the barking tree frog. A recent study in Panama using the túngara frog, *Physalaemus pustulosus*, found that *Corethrella* spp. preferred a complex call to a simple call. Using synthetic components of the barking tree frog call, a laboratory colony of Nearctic *Corethrella appendiculata* was tested in a Y-maze. The data confirmed that *C. appendiculata* was more attracted to a frequency-rich sound and not to a sound that resembles their wing-beat frequency. For the first time, the hosts of corethrellids were identified to species based on blood meal analysis. Using flies collected over six years at two locations in the SE USA, the host species data were compared to potential host abundance. Host feeding patterns did not reflect host attraction behaviors. Overall, phonotactic behaviors were observed in *Corethrella* spp.; however, the role of these behaviors in host feeding behavior remains unclear.

INDEX WORDS: Corethrellidae, Diptera, Phonotaxis, Blood feeding, Anura
HOST ATTRACTION AND HOST SELECTION IN THE FAMILY
CORETHRELLIDAE (WOOD AND BORKENT) (DIPTERA)

by

JEREMY V. CAMP

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CHAPTER 1

INTRODUCTION

Background

The family Corethrellidae has 62 extant species worldwide in the single genus *Corethrella* Coquillett (Borkent 1993). At least five species of these small, nematocerous flies, often called “phantom midges,” are endemic to North America (A. Borkent, personal communication). Most species are found in tropical or subtropical climates (Borkent 1993). Adult female *Corethrella* have biting mouthparts and are telmophagous (McKeever 1986); they are thought to feed exclusively on the blood of frogs and some are known to be facultatively autogenous. Immature stages are aquatic; species can be found in small bodies of water (e.g., epiphytes, tree holes, waste tires, roadside ditches, puddles) or at the edges of large bodies of water, particularly where there is emergent vegetation (Williams and Edman 1968; Chapman *et al.* 1971; McKeever 1977; McKeever and French 1991a). *Corethrella* larvae are predaceous on other aquatic organisms (e.g., mosquito larvae, nematodes, etc.) and have prehensile antennae and a prothoracic proleg which are used to facilitate feeding (Borkent and McKeever 1990; L.P. Lounibos, personal communication).

In 1968, the first observance of blood meals in both *Corethrella brakeleyi* (Coquillett) and another novel species raised the interest in this genus considerably (Williams and Edman 1968). Alan Stone (1968) named the new species *Corethrella wirthi* Stone, and the hosts of the three individual midges were putatively identified as mammals and birds using serological tests (Williams and Edman 1968). A decade later, McKeever (1977) observed *C. brakeleyi* and *C. wirthi* feeding on tree frogs (Anura:
Hylidae). Subsequent testing showed that the midges were attracted to the calls of certain species of tree frogs, and that the midges could be captured in great numbers by playing a recording of calling male *Hyla* spp. (McKeever and Hartberg 1980; McKeever and French 1991a).

These findings have encouraged further research on *Corethrella*: new Australian species have been described (McKeever 1988; Colless 1994); the Nearctic distribution has been extended north and west (Ellis and Wood 1974; Lund 2000) and new species have been named from the neotropics (Bernal *et al.* 2006) and Asia (A. Borkent, pers. comm.); the reproductive systems and mouthparts have been well documented (McKeever 1985a, b; McKeever 1986); and fossil specimens have been discovered (Borkent and Szadziewski 1992; Szadziewski *et al.* 1994; Szadziewski 1995). Additionally, the group has been removed from the family Chaoboridae and placed into the monophyletic clade, Corethrellidae, a sister group of the remaining Chaoboridae plus Culicidae, primarily based on the morphology of immatures (Wood and Borkent 1989).

To date, much remains unknown about this family. Aspects of their life histories remain unknown (*e.g.*, temporal patterns of emergence and abundance, over-wintering habits, mating behaviors, *etc.*). Several other aspects of the life history are unclear or unknown; most importantly, definitive unbiased evidence of host-feeding behavior does not exist.

**Significance**

Much is known about intraspecific acoustic communication, especially in the form of sexual signaling. There are few studies into the use of sound as an interspecific signal. Most of these studies focus on the exploitation of the acoustic sexual signals of
prey species by predators (e.g., Tuttle and Ryan 1981). Even though acoustic signals are often necessarily loud and conspicuous, it seems that few predators take advantage of them to locate prey. It is equally possible that information is lacking because phonotaxis typically is not investigated in predator-prey systems. Since it is known that corethrellids are attracted to the sounds of their prey, it is of importance to understand their host-seeking behavior.

In addition to the use of sound as an attractant, further study of the family Corethrellidae would be beneficial to many biological disciplines. The documented transmission of a trypanosome (Johnson et al. 1993) and the inherent quality, via hematophagy, to serve as a vector for other pathogens may alter or otherwise affect populations of one of nature’s indicator groups, anurans. In addition, it is possible that corethrellids are involved in enzootic transmission cycles of medically important viruses; for example, there is recent evidence of anuran blood meals in mosquitoes at an Eastern Equine Encephalomyelitis virus focus (Cupp et al. 2004) and Leopard frogs (Rana pipiens Schreber) in Canada have tested positive for Western Equine encephalitis virus (Burton et al. 1966).

The phylogenetic position of corethrellids, the sister group of mosquitoes, allows investigations into mosquito phylogenies (Pawlowski et al. 1996). Because both groups are hematophagous, it is likely that hematophagy initiated their common ancestor. Therefore studies into the evolution of blood-feeding in mosquitoes must include corethrellids. The oldest extant corethrellid is presumed to be C. novazealandiae and is found sympatrically with the oldest lineage of extant frogs, Leiopelma, in New Zealand (Borkent and Szadziewski 1992; Pough et al. 2004). Because of their synchronous
emergence with anurans, hematophagy in the corethrellids and in the culicids may have initiated upon amphibian hosts. Extant species in these taxa that feed on amphibians may most closely resemble the primitive (ancestral) character state. Physiological and behavioral adaptations associated with taxon-specific (e.g., amphibian) blood feeding may be informative in constructing appropriate phylogenies of these related groups.

Knowledge of the Corethrellidae is also significant for applied reasons. Corethrellid larvae are known to feed on larval mosquitoes (McLaughlin 1990) and other aquatic zooplankton. In sampling waste tire piles in Florida, significantly less container-breeding mosquito larvae were found when corethrellids were present (Morris and Robinson 1994). This may suggest that the larvae could be effective in biological control of pestiferous container-breeding mosquitoes.

Additionally, larvae of Corethrella appendiculata, a facultatively autogenous, tree-hole breeding species, were found to alter the species composition of a tree-hole community. In laboratory tests, C. appendiculata larvae preferentially consumed an invasive mosquito larvae (Aedes albopictus) over native mosquito larvae, (Ochlerotatus triseriatus) (Griswold and Lounibos 2006). Due to the relative ease of maintaining a colony of C. appendiculata and the species’ role as a predator, investigations of ecological significance are facilitated (e.g., indirect trophic interactions and their effects on community structure, invasive species survival). For these reasons and others, more must be known about the basic life histories of Corethrella species.
Recent Advances and Objectives

Circumstantial and anecdotal evidence indicates that corethrellids preferentially feed on tree frogs. This claim is supported by studies of trypanosomes of tree frogs, in which corethrellids were implicated as vectors. The trypanosomes were found only in the blood of the male tree frogs, *Hyla cinerea* (Schneider), and not in the silent female frogs (Johnson *et al.* 1993). In contrast, the first blood meals tested were identified as avian and mammalian. However, the identification used only anti-mammalian and anti-avian sera on a relatively miniscule blood sample (Williams and Edman 1968).

In prior studies, I have collected five times as many corethrellids using a sentinel flock of zebra finches as an attractant in combination with an upward-blowing fan trap (the trap was designed to collect blood-fed mosquitoes) than using frog calls as an attractant (unpublished data). Additionally, blood meals from corethrellids can be obtained during periods of sparse or sporadic hylid breeding, even from species that are not calling (personal observation), suggesting that frog calls may not be the principal attractant, even if the flies are feeding solely on frogs. More confounding is the discovery by Mangold (1978) that corethrellids are attracted to the call of the southern mole cricket (*Scapteriscus borellii*, Orthoptera: Gryllotalpidae), another prominent crepuscular/nocturnal “noise-maker.” I have also had success collecting corethrellids using both recorded rock music and bird calls.

In 2006, two independent studies attempted to provide additional insight to the phonotactic behavior of the flies. First, Borkent and Belton (2006) published data that they believed to be “the first observation of female mosquitoes being attracted by the sound of a host.” In attempts to collect *Corethrella* species, they collected 79
Uranotaenia lowii Theobald (Diptera: Culicidae) females from CDC light traps with the
light removed and with a speaker playing the call of the barking tree frog, Hyla gratiosa
LeConte. The paper included an analysis of the call of the barking tree frog, which has a
fundamental frequency of approximately 450 Hz (see also Oldham and Gerhardt 1975).
The authors note that both Corethrella species and Uranotaenia species are of
approximately the same size and wing length (wing lengths 0.7 – 2.1 mm and 2.0 – 2.3
mm, respectively). Therefore, they reason that the antennae of these species resonate
from 400 – 500 Hz, because “resonant frequencies are related to wing length and
wingbeat frequencies.” This, they conclude, may explain why the barking tree frogs’
calls are attractive.

Additionally, phonotaxis of Corethrella species was studied in Panama, and an
alternative explanation of host attraction was presented (Bernal et al. 2006). The calls of
male túngara frogs, Physalaemus pustulosus (Cope), are known to consist of a whine (a
simple, frequency-modulated call) to which chucks (multiple frequencies broadcast
simultaneously, i.e., broad-band harmonics) may be added. It has been shown that female
túngara frogs prefer whines with chucks over whines alone (i.e., prefer complex calls to
simple calls) (Ryan 1980). It has additionally been shown that predatory frog-eating bats
(Chiroptera: Phyllostomidae) are also preferentially attracted to complex calls of the
males of this species (Tuttle and Ryan 1981). Bernal et al. (2006) used both natural and
synthetic calls of túngara frogs and found that corethrellids prefer a complex call (whine
with chuck) rather than a simple call (whine only).

Taken together, these studies present two testable, alternative hypotheses
concerning host preference in corethrellids: preference for resonant frequency versus
preference for complex calls. The call of the male barking tree frog is the most attractive
call to sympatric corethrellids (McKeever and French 1991a); it is also attractive to
Corethrella species in the Western United States, Mexico, and Costa Rica, where the
barking tree frog has never been heard before (Borkent and Belton 2006). This may
support the Resonant Frequency hypothesis of Borkent and Belton (2006). However, the
call of the male barking tree frog has broad-band harmonics (Borkent and Belton 2006;
Oldham and Gerhardt 1975), similar to the chucks of the túngara frog (Bernal et al.
2006). This supports the Complex Call hypothesis of Bernal et al. (2006.) The call of
the barking tree frog, therefore, is amenable to investigating which component is more
attractive to corethrellids.

Both field and laboratory testing of the attractiveness of various sounds to the
flies are required for investigating host-seeking behavior in corethrellids. While an
investigation of the hypothetical basis for host attraction is complementary, it is
imperative to correlate this with the behavior of Corethrella species in nature. The most
attractive sounds may not be encountered during every foraging session. Therefore, it is
essential to also identify the actual hosts on which the flies are feeding. Although there is
significant evidence suggesting corethrellids are attracted to frog calls, it is unclear how
important frogs are as hosts overall.

A study on host-feeding is facilitated by analysis of blood meals taken by flies
which have been collected from natural habitats in an unbiased manner. Such a study
will not only provide the first definitive account of species fed upon by corethrellids, but
will also provide insight into which calls are more attractive on a given night of foraging
(e.g., the more complex calls, the calls nearest the resonant frequency, the more abundant
calls, the loudest calls, etc.) I will present data taken from sound attractant experiments as well as blood meal identifications in an attempt to better explain the interaction between corethrellids and their hosts.
CHAPTER 2
HOST ATTRACTION

Introduction

In the culicomorphs, locating an appropriate host typically is performed by navigating a hierarchy of increasingly specific cues (Clements 1999). The majority of these cues are associated with the detection of volatile chemicals produced by the host, which evaporate at normal temperatures and pressures, and are carried on air currents. For example, carbon dioxide, a byproduct of cellular respiration released in large quantities by endothermic vertebrates, is the principal attractant used in many mosquito traps (Service 1993). While it is unknown whether corethrellids are using such chemical cues, it is apparent that they are attracted to sound; particularly the sounds of their putative hosts – anurans.

Male anurans attract potential mates by a characteristic vocalization (Pough et al. 2004). Receptive female frogs are able to locate conspecific males by this call and may even assess the fitness of a potential mate based on certain aspects of the call. For example, gray tree frog (*Hyla versicolor*) males that have a longer call produce tadpoles that are superior foragers (Doty and Welch 2001). It has been shown that this signal is also being utilized by potential predators and parasites (Zuk and Kolluru 1998).

In the case of anurans, the calls of various species have been shown to attract snapping turtles (*Chelydra serpentina*), frog-eating bats (*Trachops cirrhosus*), and philander opossums (*Philander opossum*) (Halliday 1980; Tuttle and Ryan 1981, Ryan et al. 1982; Tuttle et al. 1981; respectively). The discovery of the attraction of corethrellids to frog calls (McKeever 1977) provides one of the very few known cases where a blood-
feeding arthropod exhibits positive phonotaxis to its vertebrate host; other cases include
an ornithophilic soft tick (*Ornithodoros concanensis*) being attracted to the chatter of
nestling cliff swallows (Webb *et al.* 1977), sounds produced by barking dogs attracting
*Rhipicephalus sanguineus*, and the larvae of *Boophilus microplus* becoming activated
upon detecting the lowing of cattle (Sonenshine *et al.* 2002).

Frogs spend large amounts of energy producing calls that are detectable at long
distances. Therefore it is not surprising that a parasite might take advantage of such a
signal, given the proper receptors. It is known that male mosquitoes, in general, have
such receptors for the detection of the wing beat frequencies of female conspecifics. The
receptors, also called the Johnston’s organ, are located on the enlarged pedicel, and are
thought to have a resonant frequency (= structural sensitivity) that is “tuned” to the
specific frequency of the female wing beat (Göpfert *et al.* 1999; Clements 1999). It has
recently been shown that *Uranotaenia lowii* is also capable of detecting the sounds of
their frog hosts and may be using these receptors to do so (Borkent and Belton 2006). It
is not known whether *Corethrella* species have such receptors. McKeever (1988)
described a novel sensillum on the antennae of Nearctic *Corethrella* which may function
in sound detection; however, not all species of *Corethrella* have such a structure (A.
Borkent, personal communication).

In general, it is clear that corethrellids exhibit positive phonotaxis to a wide range
of sounds (McKeever and French 1991a, pers. obs.) Given the variety of the calls of
different frog species, attraction to many sounds is a suitable strategy for opportunistic
feeding behavior in *Corethrella*. Therefore it is not surprising that any sound produced at
night might attract a corethrellid (*e.g.*, playback of recorded rock music) (pers. obs.)
However, corethrellids show preference for the calls of certain species of frogs; namely, the barking tree frog, *Hyla gratiosa* (McKeever and French 1991a). It also has been documented that species of *Corethrella* can be captured in large numbers using the call of *H. gratiosa* in areas where the frog has never been recorded (e.g., Mexico, southwest United States, and Costa Rica) (Borkent and Belton 2006, McKeever and French 1991b). Additionally, field studies in Panama have shown that endemic corethrellids (which feed on the túngara frog, *Physalaemus pustulosus*) prefer the complex calls of the túngara frog over simple calls (Bernal et al. 2006).

From these accounts, two hypotheses have emerged to account for host attraction behavior in corethrellids. First, it has been suggested that the flies are preferentially attracted to the call of *H. gratiosa* because the call contains the resonant frequency of the flies’ antennae, which I will refer to as the Resonant Frequency hypothesis. Second, the Complex Call hypothesis states that corethrellids are choosing those calls that have the most complexity, characterized by a broad-band harmonic series (*i.e.*, multiple, simultaneous frequencies). This study assesses these hypotheses using corethrellids native to the southeastern United States in field and laboratory settings in an attempt to provide a background for future research. Additionally, field experiments were performed to replicate the findings of McKeever and French (1991a).

**Methods**

**Study Site.**

Experiments were carried out primarily at Bird Pond, a large pond (approximately 2.02 hectares) located on the southern border of the city of Statesboro in Bulloch County, Georgia, USA (32° 23’ 51” N, 81° 46’ 17” W) (Figure 2.1). The pond was created by the
damming of Little Lotts Creek and serves the city as a storm water catchments facility. The northern half of the pond is flooded woodland that was recently re-colonized by beavers. The southern half of the pond is open water habitat with several shallow inlets covered with emergent vegetation including various grasses and reeds. Descriptions of the ideal habitat for eastern Nearctic Corethrella spp. (Williams and Edman 1968; Chapman et al. 1971; McKeever 1977; McKeever and French 1991a; pers. obs.) indicated that this was an ideal habitat and preliminary trapping confirmed the presence of corethrellids.

**Sampling techniques.**

To test the attractiveness of various sounds, miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) were set at dusk. The traps were placed 0.5 m above the ground, 0.5 m from the shore, and spaced approximately 50 m apart at several sites around Bird Pond. Lights were removed from the traps and recorded sounds were broadcast over the intake of the trap using a small, portable cassette tape recorder (GE® Model 35027 AC/DC Cassette Recorder). The speaker of the cassette tape recorder was pointed toward the nearest body of water (see McKeever and Hartberg 1980 for photographed trap configuration). The traps were set before sunset and were turned on approximately 30 minutes after sunset. Because cassette tapes were used, the 90-minute tapes had to be “flipped” every 45 minutes. Prior trappings indicated that corethrellids can easily pass through the wire cloth affixed to the bottom of the standard CDC Light trap collection cups. To prevent any loss of specimens a fine mesh nylon hose was placed around the bottom of the collection cup.
At the end of each trapping session, the collection cups were taken to a laboratory and placed in a freezer at \(-20^0\text{C}\) to kill any arthropods. Materials from the cups were then sorted and identified to species on a chill plate using a binocular dissecting microscope (see Stone 1968 for keys to Nearctic female *Corethrella* species). All corethrellids were kept in small, glass, screw-top vials and stored at \(-20^0\text{C}\); mosquito specimens were identified to species and recorded but specimens were discarded. Any blood-fed corethrellid specimens were placed in individual autoclaved 1.5 mL microcentrifuge tubes and stored at \(-70^0\text{C}\) until blood meal identifications could be performed.

**Replication of McKeever and French experiment.**

Nine species of summer-breeding frogs endemic to the southeastern United States were chosen as treatments. The calls of the species were obtained from a commercially available set of recordings (Elliot 2004) and were recorded onto a 90 minute cassette tape. The taped recordings consisted of 30 seconds of an individual frog’s advertisement call followed by 30 seconds of silence; this was repeated for the duration of the 90 minute cassette tape.

The frog call tapes were grouped into three cohorts: two cohorts of summer breeding tree frogs (hylid cohort 1: *Acris gryllus, Hyla chrysoscelis, Hyla avivoca*; hylid cohort 2: *Hyla gratiosa, Hyla cinerea, Hyla squirella*) and one cohort of summer-breeding true frogs (Ranidae) (ranid cohort: *Rana catesbeiana, Rana grylio, Rana clamitans*). Each cohort was played with a fourth trap which broadcast the call of a non-endemic bird, the Common Raven (*Corvus corax*, Corvidae: Passeriformes). This was done as a form of control since corethrellids are not known to feed on birds. Each cohort
was played for three hours a night such that one night of trapping equals four trap nights. Each cohort was played for three nonconsecutive nights at irregular intervals from June to September 2005 (36 total trap nights).

Each cohort was analyzed using a generalized Poisson loglinear model (PROC GENMOD, SAS Institute) and calculating goodness of fit statistics (Wald $X^2$-test). The means and 95% standard errors were used to determine relative attraction. These statistics may be large due to sampling method; therefore it was beneficial to calculate a dissimilarity index (Agresti 1996). Cohorts were combined and compared using Wald 95% confidence intervals to determine the most attractive frog call overall.

Development and testing of a synthetic barking tree frog call.

The call of the barking tree frog (*H. gratiosa*) was analyzed using the Raven software package from the Cornell Laboratory of Ornithology (Charif *et al.* 2003) (Figure 2.2). Properties of the call were replicated using NCH Tone Generator software available from NCH Swift Sound. The software allows desired frequencies (in Hz) and relative amplitudes (in dB) to be entered and played for a specified duration. After determining that 420 Hz was the fundamental frequency of the barking tree frog call, the following six frequencies were used to generate the synthetic call: 420 Hz, 840 Hz, 1260 Hz, 1700 Hz, 2120 Hz, and 2540 Hz. The highest amplitude was given to the fundamental frequency and the overtone frequencies were assigned amplitudes relative to the fundamental frequency as follows, respectively: 30 dB, 20 dB, 10 dB, 20 dB, and 30 dB below the fundamental frequency.

The created tone (Figure 2.3) was analyzed using Raven software package to ensure its likeness to the barking tree frog call before being digitally recorded. This
recording was than used to make a 90 minute cassette tape; the 400 millisecond sound played once every six seconds for the duration of the tape. The same individual frog’s call that was used to create the synthetic call was recorded onto a 90 minute cassette tape and played at a rate of once every six seconds for the duration of the 90 minute cassette tape.

Trapping proceeded in the manner described above (see “Sampling Techniques”) with a third trap that contained a blank 90 minute cassette tape acting as a negative control. For this experiment, a full-factorial design was used to control for the time of night and site-specific environmental factors that may confound the results. Three sites were chosen at the south end of the pond. On a given night, each call was played from each site. In addition, collection cups were harvested each time the calls were rotated, at approximately 2200, 2300, and 2400 hours (EDT), and replaced with new collection cups. Trapping was performed on nine nights from 11 July 2006 through 16 August 2006 to satisfy the full factorial design (i.e., each call was played at all three times, at all three sites, during the course of nine nights). Trapping was also done for four additional nights at a different location at Bird Pond; treatments were arbitrarily assigned to traps and the traps were operated for three hours after sunset.

The full-factorial design was analyzed using a 3 X 3 X 3 contingency table and calculating Fisher exact test statistics (PROC FREQ, SAS Institute). Confounding variables (site and time of night) were tested for conditional independence from the treatments and response using Cochran-Mantel-Haentzel statistics (PROC FREQ, SAS Institute). A Wilcoxon Signed Ranks test was performed to test whether time of night or site could explain variation in collections. A paired t-test was performed combining
results from the full-factorial experiment and the four additional nights of trapping; the control trap data were omitted and the two treatment traps were compared for their difference. This analysis treats each fly collected as an independent datum, for which precedence has been set (Ulagaraj and Walker 1973; Walker 1993). Additionally, to quantify the difference between treatments, an exact, two-tailed binomial probability was calculated using pooled data to determine if flies were choosing randomly.

Laboratory testing of complex vs. resonant sounds.

Collections to test whether corethrellids prefer a complex sound (i.e., frequency-rich harmonic overtones) or a simple sound (i.e., a sound that most closely matches their wing beat frequency) were initially begun in field tests similar to those described above. However, the experiment was moved to the Florida Medical Entomology Laboratory (FMEL) in Vero Beach, Florida, USA after three consecutive nights of field trapping yielded small numbers of corethrellids (Table 2.1). The FMEL maintains a laboratory colony of Corethrella appendiculata at 25 ± 1°C with a light:dark regime of 14:10 hours and relative humidity of ~70%. Larvae were reared to 4th instars on a diet of cultured nematodes (Lounibos, unpublished). At the time of the experiment (9 October 2006) the colony consisted of approximately 300 adults at varying times since eclosion.

A crude Y-maze was assembled to offer individual midges a choice between sounds (Figure 2.4). A plastic 2 liter soft drink bottle was used as a central chamber; the top and bottom were removed and a small hole was added to the center to aid in the addition and removal of flies. To each end of the central chamber fine mesh nylon hoses were attached, the nylons were held open by a wire frame that created a tube 6 cm in diameter and 20 cm long. In order for a fly to pass from the central chamber to either
mesh tube it had to pass through a narrow funnel (diameter = 1.5 cm). This was created by closing the fine mesh with a wire near the mesh tube. In this way, flies that were attracted to the sound were directed into the outer tubes by the funnel, while making it difficult for flies to move into the central chamber from either tube (there was no funnel leading into the central chamber). Small cassette tape players broadcast the sounds towards the center chamber from the 20 cm outside of either end of the maze. A midge was recorded as choosing a sound when it passed through the funnel into the wire-framed tubes. In this manner, only receptive females (i.e., midges that chose a sound) were used in statistical analyses.

The experiment was performed immediately after the dark cycle began (approximately 2100 hr, EDT). Ten female flies were chosen at random and were added to the central chamber and allowed to sit in darkness for 10 minutes with no acoustic stimulus; the number of flies in each chamber was then recorded. Immediately following this, any flies in the outer tubes were placed back into the central chamber. The cassette players then began broadcasting the sounds in darkness for ten minutes while the researcher was out of the room. Afterwards the funnels were closed and the number of flies in the central chamber and in each tube was recorded; the flies were placed in a cage separate from the colony to ensure that individuals were only tested once. This cycle of 10 min. of acclimation followed by 10 min. of acoustic stimuli was repeated ten times.

The sounds used were generated with NCH Tone generating software (NCH Swift Sound). One sound was a simple call (a single frequency, 420 Hz) (Figure 2.5), the other sound was a complex call (the harmonic overtones present in the barking tree frog’s call excluding 420 Hz) (Figure 2.6). Components of the experimental setup were rotated as
much as possible (i.e., tapes were transferred to alternate cassette tape players, the side
from which the tape was broadcast was changed, the maze, itself, was rotated 90\(^\circ\)) in an
attempt to eliminate the influence of unknown, confounding stimuli.

Additionally, flies were tested for their preference for a synthetic barking tree frog
call (see previous subsection) versus a simple call (420 Hz) using the same maze. Only
female flies were used; five subsets of ten flies were allowed 10 min. of acclimation
immediately followed by 10 min. of acoustic stimuli. Again, components of the
experimental setup were rotated as much as possible.

The data were analyzed by computing a G-test comparing the number of flies
found in each chamber during the acclimation periods to the number of flies found in
each chamber during the acoustic stimuli periods. Additionally, exact two-tailed
probabilities of a binomial distribution were calculated for flies recorded as “choosing”
an acoustic stimulus to determine if more flies were attracted to a stimulus than expected.
In this analysis, flies that remained in the central chamber were considered to be
unreceptive (e.g., were not engaging in host-seeking behavior due to reproductive status)
and were excluded from the analysis. Finally, paired \(t\)-tests were performed comparing
flies recorded as “choosing” a given stimulus over the alternative to control for variation
between experimental groups. Again, this analytical method treats each fly as an
independent datum.
Results

Replication of McKeever and French experiment.

Over forty trap nights, 637 corethrellids were caught: 454 (71.3%) *Corethrella brakeleyi* and 183 (28.7%) *Corethrella wirthi*. The fewest flies captured by a single trap night was a single fly being attracted to the call of *Rana clamitans*; the call of the Common Raven captured the largest number of flies on a single trap night (*n* = 67). All traps captured flies at an average of approximately 16 flies per trap night. The cohort containing *Acris gryllus*, *Hyla avivoca*, and *Hyla chrysoscelis* (hylid Cohort 1) and the cohort containing *H. cinerea*, *H. gratiosa*, and *H. squirella* (hylid Cohort 2) each attracted 187 flies. The ranid cohort attracted 180 flies (*Rana catesbeiana*, *R. grylio*, and *R. clamitans*).

For each cohort, loglinear model provided a poor fit (Wald Type 3 statistics: hylid Cohort 1, \(X^2=1571.69, \text{d.f.}=4, \ p<0.0001\); hylid Cohort 2, \(X^2=2159.4, \text{d.f.}=4, \ p<0.0001\); ranid cohort, \(X^2=2107.44, \text{d.f.}=4, \ p<0.0001\)). Introducing other predictors, such as trap site and trap night, did not improve the model significantly. The dissimilarity indices show that hylid Cohort 1 provides a better fitting model (\(D=0.294\)) than hylid Cohort 2 or the ranid cohort (\(D=0.46, 0.44\), respectively), where a score of zero indicates a perfectly-fitted model. The Pearson Chi-squared test also confirms hylid Cohort 2 providing the best fit of these (\(X^2=11.7, \text{d.f.}=4, \ p=0.019\)).

Due to this lack of fit, only generalized inferences may be made for the data. In hylid Cohort 1, *H. chrysoscelis* attracted more corethrellids than any other call, while the other calls attracted approximately the same number of flies (Figure 2.7). It is clear that in hylid Cohort 2, *H. gratiosa* attracted the most corethrellids (this can be seen from the
non-overlapping confidence limits calculated from the model) (Figure 2.7). Finally, in the ranid cohort, \textit{R. clamitans} attracted few corethrellids and the Common Raven (pseudo-control) collected the most corethrellids (Figure 2.7). Overall, \textit{H. gratiosa} attracted the most flies (\(n=99; 21\%\) of total flies) (Figure 2.7)

**Development and testing of synthetic barking tree frog call.**

A total of 127 corethrellids were captured over 27 trap hours during 9 trap nights at an average of 4.4 flies per trap hour. Twenty (8\%) \textit{Corethrella wirthi} were collected and 213 \textit{Corethrella brakeleyi} were collected (92\%). However, seven trap hours collected no corethrellids: three control traps, three real barking tree frog call traps, and one synthetic barking tree frog sound trap (Table 2.2). This contributed to lack of fit of statistical models that contained the predictors: site and time of night. It can be noted that all partial tables tested indicated conditional dependence at all levels of each controlled variable (CMH tests, \(p<<0.001\)) (PROC FREQ, SAS Institute 2004). Fisher’s exact tests yielded low one-tailed p-values (\(p<<0.0001\)), indicating an unlikely outcome (and a poor fit). However, this is primarily due to the traps in which no flies were caught contributing to large residuals. Taken separately, there was no significant difference in collections between time of night (Wilcoxon Signed Ranks, \(X^2=1.80, p=N.S.\)) nor between trap sites (Wilcoxon Signed Ranks, \(X^2=1.36, p=N.S.\)) (JMP, SAS Institute 2006).

Control traps always collected much less than half the amount of corethrellids as either sound trap; therefore the control trap was eliminated from analysis of the effectiveness of the sound traps. Twelve additional trap nights were added to the aforementioned nine trap nights, catching an additional 106 flies. A paired t-test revealed
that there exists a significant difference between the two calls, controlling for trap night
\((t=8.44, p=0.0011)\) (Table 2.3). The synthetic call attracted 128 *Corethrella* and the real
call attracted 84 *Corethrella*, a two-tailed binomial probability \((p = 0.003)\) indicates that
the flies preferred the synthetic call over the real call.

**Laboratory testing of complex vs. resonant sounds.**

Taking all 10 test periods together, five flies were recorded on the side of the trap
nearest the simple sound, 25 were recorded on the side of the complex sound, and 70 flies
remained in the central chamber (Table 2.4). For the test of the synthetic barking tree
frog call versus the simple sound, the totals of the 5 test sessions were as follows: 6 flies
were recorded on the side of the trap nearest simple sound, 14 were recorded on the side
of the synthetic call, and 30 remained in the central chamber (Table 2.5). When the
sounds were being played, corethrellids that had “chosen” a side showed increased
activity, hopping around the chamber whereas those remaining in the central chamber
were relatively motionless.

The distributions of flies in the chambers when acoustic stimuli were offered were
found to be significantly different than during the acclimation period for both
experiments: simple sound versus complex sound \((G\text{-test}, X^2=19.0, \text{ d.f.}=2, p < 0.001)\),
and synthetic call versus simple sound \((G\text{-test}, X^2=33.0, \text{ d.f.}=2, p < 0.001)\). For the tests
of the simple sound versus the complex sound, a paired \(t\)-test revealed that the means
were significantly different, controlling for trap session \((t=3.58, p=0.006)\); with more
flies found nearest the complex sound. For the tests of the simple sound versus the
synthetic call, it was found that the means were not significantly different, controlling for
trap session (paired \(t\)-test; \(t=2.36, p=0.078\)). Excluding the flies remaining in the central
chamber, the exact two-tailed binomial probability was calculated; more flies were found on the side of the synthetic barking tree frog call \( (p=0.04) \) when trials were pooled.

**Miscellaneous notes.**

During the 2005 and 2006 field collections a total of 15 male *Corethrella* spp. were collected from various sound traps. Females and males of several species of mosquitoes were also found in sound traps, namely *Anopheles crucians*, *An. quadrimaculatus* s.l., *Culex erraticus*, *Cx. nigripalpus*, and *Coquilletidia perturbans*, the latter three species being the most abundant. Of particular note, 8 male and 4 female *Uranotaenia sapphirina* were collected in sound traps.

**Discussion**

Three primary conclusions are suggested by these data. First, the call of the barking tree frog is a very attractive call to Nearctic corethrellids. Second, a synthetic call of the latter portion of the barking tree frog’s call is at least as good as the actual call in attracting corethrellids. Finally, it appears that it is the broad-band harmonic series that is most attractive to corethrellids, not the dominant frequency of 420 Hz. Although it could not be conclusively shown that *C. appendiculata* preferred the synthetic call over the simple sound in laboratory tests, the midges showed a clear preference for the synthetic call without 420 Hz (complex sound) over a 420 Hz sound alone (simple sound). In reference to two recently proposed hypotheses concerning corethrellid host-seeking behavior (Borkent and Belton 2006; Bernal *et al.* 2006), a complex call is more attractive than a simple call and the attractiveness of a call may not be due solely to an innate attraction to a resonant frequency of the flies antennae (*i.e.*, a frequency that matches the wing beat frequency of females).
One potential confounding effect that was not tested was the possibility of a negatively phonotactic response. This behavior has never been assessed with corethrellids; only field tests that relied on positive phonotaxis have been performed. Whether the flies exhibited a negatively phonotactic behavior was not assessed in my tests using a y-maze in a laboratory. However, the existence of this behavior would negate some of my results. A new strategy for testing flies in the laboratory would be critical to understand fully the behavior of corethrellids (e.g., testing a simple sound versus no sound).

It has been known that the barking tree frog call was the most attractive sound to corethrellids where both are endemic (McKeever and French 1991a). From an evolutionary standpoint, it is odd that this call would be attractive to corethrellids that are not sympatric with *H. gratiosa*. Surely species of *Corethrella* in Central America are not seeking *H. gratiosa* as their preferred host. It was not until recently that a solution was proposed.

In studying the relationship between corethrellids and frogs in Panama, native corethrellids preferred a complex call (Bernal *et al.* 2006). Indeed, the call of *H. gratiosa* is a complex call (Oldham and Gerhardt 1975; Borkent and Belton 2006) and it appears that it is the complex quality of the call that is attractive. Therefore, it seems that corethrellids have a stereotyped search “image” when host seeking that allows them to choose a complex call over a simple call. In this sense, it is a mere coincidence that the barking tree frog’s call is so attractive to many species of *Corethrella*, even species that have never heard a barking tree frog.
It is not clear what a more complex call indicates about a species of frogs. For example, *Hyla crucifer*, the spring peeper, is a tree frog that occurs sympatrically with the flies tested in this study. The call of the spring peeper consists of three frequencies being broadcast simultaneously, *i.e.*, a complex call. However, this species does not appear to be any more attractive to corethrellids than other species of frogs. Very little analysis has been done on the complexities of frog calls. More data need to be collected to understand why this property presumably indicates a better blood source to a corethrellid. The answer may lie in the hearing organ, which is currently unknown.

It has been suggested that the Johnston’s organ is used by species of mosquitoes and corethrellids to receive the frogs’ acoustic signals (Borkent and Belton 2006). However, it is not known if the organ functions similarly in females nor has it been shown that corethrellids possess a Johnston’s organ. It is known that structurally, the Johnston’s organ is only capable of detecting short-range sound waves (Clements 1999). An organ for the detection of long-range air-borne sound waves is known in many animals, and through convergent evolution is in the form of a tympanal ear. The only flies known to possess a tympanal ear are in the tribe Ormiini (Tachinidae: Diptera) and are parasitoids of orthopterans (Robert *et al.* 1995). Attempts to locate the hearing organ in corethrellids should focus on determining if a similar structure exists.

For the túngara frog, it is known that a more complex call is more attractive to female conspecifics (Ryan 1980). It is interesting that a complex call is also more attractive to a predator, a frog-eating bat, as well as a parasite (a “micro-predator”), the *Corethrella* spp. (Tuttle and Ryan 1981, Bernal *et al.* 2006). Unfortunately, much
remains unknown about why these three very different taxa prefer this trait and the implications this has on sexual selection in general.

Prior studies on the attraction of corethrellids to the frogs have neglected to note the important consequences from the frog’s point-of-view. Although the specifics of this system have yet to be studied, parasitism by hematophagous arthropods can be costly. In the Neotropics, a broadcast call attracted up to 500 corethrellids in 30 min.; in the southeastern U.S., 566 corethrellids were captured in the same amount of time (Bernal et al. 2006; McKeever 1977). Although the blood meals are small (pers. obs.), a conservative estimate of 0.5 µL of blood being taken by 15 flies a minute will remove 0.45 mL of blood in an hour. A 20 gram tree frog has approximately 1.6 mL of blood (Conklin 1930), making exsanguination a serious possibility in a matter of hours. Additionally, it is known that a trypanosome of frogs is transmitted by corethrellids (Johnson et al. 1993), which may impose further costs on the host.

Despite such possibility for intense parasitism, there are very few accounts of midge-deterrent behaviors. It has been noted that frogs used for a blood source in maintaining a colony of corethrellids made no attempt to consume or otherwise deter corethrellids from feeding (McKeever and French 1991a). The túngara frogs observed in Panama sacrifice calling time for swatting at the midges and attempting to wipe them off (Bernal et al. 2006). I have witnessed corethrellids on a large Cuban tree frog, Osteopilus septentrionalis, in Vero Beach, FL USA make no attempt to deter corethrellids from feeding on it. The frog was not calling but attending a light in search of insect prey. However, when an individual midge attempted to feed around the eye the frog became rigid and retracted both eyes until the midge left.
Another important question that arises from these data concerns the potentially maladaptive call of the barking tree frog. Again, the precise costs imposed by *Corethrella* spp. are not known. However, it is important to review strategies employed by *H. gratiosa*, which apparently has the most attractive call in the New World. Barking tree frogs are known to spend the daytime high in the canopy and descend to the water at night where it calls from the water (Neill 1958). In submersing most of its body perhaps it is avoiding persistent midges. In addition, barking tree frogs are some of the rarer frogs throughout their range (Conant and Collins 1998). They are infrequent callers and usually call in a chorus; duetting seems to be an important calling behavior (Neill 1958). It is not known whether satellite male behavior (silent males near calling males that attempt to intercept females attracted to the calling male) exists in this species; however, this behavior may be an ideal strategy used to avoid parasitism. In general, this system certainly warrants further study.

In addition to corethrellids, the traps collected both male and female mosquitoes. The females of the species collected, with the exception of *Ur. sapphirina*, were likely to have been attracted to me while I was flipping a cassette tape. Male mosquitoes were also captured in significant numbers, more so than might be expected from a standard CDC Light trap (it is a relatively rare occurrence, personal observation). Overall, it appears some mosquitoes are responding to acoustic stimuli, supporting evidence that *Uranotaenia* spp. in the neotropics and in Japan may be using sound as an attractant (Borkent and Belton 2006; Toma *et al.* 2005).
Problems with study.

Simple experimental designs can be far more instructive than complex designs. The design involving a simple choice provided a very clear indication of which sounds were more attractive to corethrellids. In any case, insufficient replication, lack of predictors (e.g., meteorological data), and several traps collecting no corethrellids led to the inability to make proposed statistical analyses. It has been noted that humid nights immediately following a rain are ideal for trapping species of *Corethrella* (McKeever 1977). In the field studies, trap nights were selected based on relative humidity and days since rain, although no measurements were taken. These types of data are needed to maximize future trap successes.

Using binomial distributions and paired *t*-tests provided a clearer statistical picture. It should be noted that these methods were acceptable under the assumption that all receptive female corethrellids that perceived the sounds on a given night were captured in the traps. In this way it is possible to treat each fly as an independent biological entity and disregard the variation due to other variables. However, this is not the most desirable statistical approach and limits the extent of inferences that may be made using the data.
Tables and Figures

Table 2.1. Numbers of *Corethrella brakeleyi* attracted to sound traps. Sound traps were set on three nights at three different trapping locations at Bird Pond, Statesboro, GA, USA. Pairs of traps (A, B, and C) shown were placed 5 m apart and broadcast various calls for three hours each night. F = Synthetic barking tree frog sound; C = Complex call (synthetic barking tree frog sound with fundamental frequency removed); S = Simple call (420 Hz, the fundamental frequency of the barking tree frog call).

<table>
<thead>
<tr>
<th>Trial</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>F</td>
<td>C</td>
<td>F</td>
</tr>
<tr>
<td>26-IX-2006</td>
<td>6</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>27-IX-2006</td>
<td>11</td>
<td>5</td>
<td>1</td>
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<tr>
<td>02-X-2006</td>
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<tr>
<td>Sum</td>
<td>17</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 2.2. Testing the synthetic call. The number of *Corethrella* spp. attracted to traps at different three different sites and three different times of night. The traps broadcast either a synthetic barking tree frog call, a real barking tree frog call, or a blank tape (Control). Fisher’s exact test $p < 0.0001$.

<table>
<thead>
<tr>
<th>Start Time</th>
<th>Site</th>
<th>Synthetic</th>
<th>Real</th>
<th>Control</th>
<th>Sum</th>
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<tr>
<td><strong>Sum</strong></td>
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<td><strong>70</strong></td>
<td><strong>41</strong></td>
<td><strong>16</strong></td>
<td><strong>127</strong></td>
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</table>
Table 2.3. Testing a synthetic sound at six sites. The number of flies captured at six sites using sound as a trap attractant. The sounds used were a synthetic barking tree frog call, a real barking tree frog call or a blank tape (= control). Excluding the control, there was a significant difference between the synthetic call and the real call (paired $t$-test, $p=0.0011$).

<table>
<thead>
<tr>
<th>Site</th>
<th>Trap Synthetic</th>
<th>Trap Real</th>
<th>Trap Control</th>
<th>Sum</th>
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<td>Sum</td>
<td>128</td>
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Table 2.4. Laboratory choice tests of simple versus complex sounds. Flies attracted to sounds as tested using a simple y-maze using a laboratory colony of *Corethrella appendiculata*. The simple sound was a single frequency of 420 Hz; the complex sound was a synthetic barking tree frog call that included only the five overtones, not the fundamental frequency (420 Hz). *N.R.* = flies not responding to sounds. There was a significant difference between the attractiveness of the simple vs. the complex sound (paired *t*-test, *p* = 0.006).

<table>
<thead>
<tr>
<th>Trial</th>
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<th>Complex</th>
<th>N.R.</th>
<th>Sum</th>
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<tr>
<td>Sum</td>
<td>5</td>
<td>25</td>
<td>70</td>
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</table>
Table 2.5. Laboratory choice tests of simple versus synthetic sounds. Flies attracted to sounds as tested using a simple y-maze using a laboratory colony of *Corethrella appendiculata*. The Simple sound was a single frequency of 420 Hz; the Synthetic sound was a synthetic barking tree frog call included a fundamental frequency of 420 Hz and five overtones. *N.R.* = flies not responding to sounds. There was not a significant difference between the attractiveness of the simple vs. the synthetic sound (paired *t*-test, *p* = 0.078).

<table>
<thead>
<tr>
<th>Trial</th>
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<td>14</td>
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Figure 2.1. Map of Bird Pond.

Bird Pond, Statesboro, Bulloch County, Georgia, USA
Figure 2.2. Spectrogram view of the mating call of the barking tree frog. Frequency (in kilohertz) is depicted as a function of time (in seconds); the relative intensity of each line indicates relative sound pressure level, the darkest lines are the loudest.
Figure 2.3. Spectrogram of a synthetic barking tree frog call. Six frequencies were generated at varying sound pressure levels to replicate the latter half of the mating call of the barking tree frog, *Hyla gratiosa*; the sound has a 420 Hz fundamental frequency and five overtones. Frequency (in kilohertz) is depicted as a function of time (in seconds); the relative intensity of each line indicates relative sound pressure level, the darkest lines are the loudest.
Figure 2.4. Diagram of Y-maze used in laboratory choice tests. Female flies were placed into the central chamber (A) by aspiration through a closable port (B). Sounds were played through speakers (C) at either end of the maze. Flies were recorded as having chosen a sound when they passed through the gates (D) and entered the side chambers (E). The side chambers (E) were made of a wire frame surrounded by fine mesh nylon hoses; the central chamber (A) was constructed from a clear 2 liter plastic bottle with both ends removed.
Figure 2.5. A simple, 420 Hz, sound. The sound was created using NCH tone generating software and is analyzed here in a spectrogram using Raven interactive sound analysis software. Frequency (in kilohertz) is shown as a function of time (in seconds).
Figure 2.6. A complex sound. The sound was created using NCH tone generating software and is analyzed here in spectrogram view using Raven interactive sound analysis software. This sound consists of the overtones present in the synthetic and real barking tree frog call at the same frequencies at the same relative sound pressure levels. The fundamental frequency (420 Hz) of the barking tree frog call is not included.
Figure 2.7. Number of Corethrella attracted to frog calls. Ag = the mating call of Acris gryllus; C1 = control, the Common Raven; Ha = the mating call of Hyla avivoca; Hch = the mating call of Hyla chrysoscelis; C2 = control, the Common Raven; Rca = the mating call of Rana catesbeiana; Rcl = the mating call of Rana clamitans, Rgr = the mating call of Rana grylio; C3 = control, the Common Raven; Hcn = the mating call of Hyla cinerea; Hsq = the mating call of Hyla squirella, Hgr = the mating call of Hyla gratiosa. Lines divide the chart into three cohorts; calls within the lines were tested on the same nights (3 nights per cohort). Bars indicate the mean number of flies with ± 95% Wald Confidence Limits.
CHAPTER 3
HOST SELECTION

Introduction

The insect order Diptera contains twelve families of hematophagous flies, including the family Corethrellidae (Wood and Borkent) (Hall and Gerhardt 2002). Medically important families of Diptera (e.g., the Culicidae) are relatively well-studied; corethrellids are not. When determining the vertebrate hosts of these flies, particularly when hosts may be from several vertebrate taxa, the method that provides the least biased evidence is the identification of host blood in the midgut of a blood-fed fly collected from a natural habitat. In the case of members of the family Corethrellidae, this has only been performed to a very limited extent - three flies tested by Williams and Edman (1968).

Both anecdotal accounts and their phonotactic response to sound indicate that the primary hosts of corethrellids are anurans (McKeever 1977; McKeever and Hartberg 1980; McKeever and French 1991a; Borkent and Belton 2006; Bernal et al. 2006). However, as mentioned previously, the first blood meals tested were identified as avian and mammalian (Williams and Edman 1968). These identifications were made using antisera that was manufactured to identify vertebrate hosts to class (in general) in a capillary tube reaction. The authors noted that, because the corethrellid blood meals reacted to both avian and mammalian antisera, the identifications were somewhat tenuous (Williams and Edman 1968).

Identifying the host species being fed upon by corethrellids is essential to understanding their life history and ecological role. There is evidence that corethrellids prefer the calls of some species of frogs over others, particularly the barking tree frog,
Hyla gratiosa (McKeever and French 1991a). The feeding strategy of corethreilids is not known; their preference for certain species may indicate specialization. However, if the most attractive host (a tree frog) is not available (as is the likely case in much of their Nearctic range), corethreilids may adopt an opportunistic strategy. The data presented in the previous chapter and by others (Bernal et al. 2006) indicates that corethreilids have a stereotyped “search image” for a complex call. The actual host feeding patterns are essential to providing a better understanding of this system.

This study presents, for the first time, specific identification of hosts of corethreilids collected over six years. In addition to presenting host feeding patterns, potential hosts in situ were identified, their abundances noted, and properties of their calls were analyzed.

Methods

Collection of blood meals.

From March through October, 2001-2004 corethreilids were collected during a Eastern Equine Encephalomyelitis virus surveillance project at an enzootic focus in Tuskegee National Forest, Macon County, Alabama, USA (32° 25’ 50” N, 85° 38’ 40” W). The site and methods for the entire study have been described previously (Cupp et al. 2003; Hassan et al. 2003; Cupp et al. 2004); here, discussion will be limited to the collection and processing of Corethrella spp. Nine resting boxes (Edman et al. 1968) and six natural resting sites (rodent burrows) were sampled twice a week in the morning with a modified CDC backpack aspirator (Model 1412; J.W.Hock Co.) Insects were anesthetized with CO₂ gas and collection cup contents were sorted on a chill table (BioQuip Products, Inc., Rancho Dominguez, California, USA) to species. Individual
blood fed female corethrellids were placed into 1.5 mL autoclaved microcentrifuge tubes and stored at -70°C. The flies remained at -70°C until DNA extractions were made in 2006. The anuran fauna at the site was noted and recorded from 2002-2004 in the form of species presence/absence.

During the summers of 2005 and 2006 corethrellids were collected from nine modified resting boxes using a modified CDC backpack aspirator with a modified collection cup at Bird Pond near Statesboro, Georgia, USA (see Chapter 2 for site description; Figure 2.1). Resting boxes were constructed according to Edman et al. (1968), however the bottom panel was absent and the boxes were deeper. The resting boxes were placed at least 15 m apart near the banks of the west side of the pond facing west. Resting boxes were sampled at irregular intervals from June through September of each year (11 times in 2005, 12 times in 2006).

The collection cup was modified by placing a fine mesh nylon hose inside the cup such that, when the aspirator suction was applied, the end of the nylon spread to fit the inside of the cup. After aspiration of the resting box, with the aspirator running, the nylon hose was carefully removed and the open end was clasped shut. A separate nylon hose was used for each box and the hoses were identified by a small label being placed inside. Insects were anesthetized by placing them at -20°C for one minute and the contents of the hoses were then sorted on a chill table (BioQuip). Individuals were placed into 1.5 mL autoclaved microcentrifuge tubes and stored at -20°C for up to three months before being transferred to -70°C.
Sampling of anuran fauna.

Several methods were used to assess the population of anurans present at Bird Pond. Visual encounters with species of anurans were recorded and their sex was determined when possible. Fifteen artificial refugia were constructed out of polyvinyl chloride (PVC) pipes (Moulton et al. 1996). The pipes were approximately 1 m in length and were closed at one end. A 2 cm hole was drilled approximately 15 cm from the sealed end to drain rain water. The pipes were hung on trees with the tops 2 m from the ground at locations near the resting boxes, usually over water. The refugia were checked every time resting boxes were sampled and the number and species of frogs were noted.

Intensive systematic assessment of anuran densities and distributions at the site was focused primarily on audio detection of calling male frogs. The relative number, location, and species of calling frogs were recorded for three one-minute intervals on nights prior to resting box sampling. The three sampling intervals were conducted at sunset and at one and two hours past sunset and three different sites near the resting boxes.

For statistical analyses of anuran diversity, a score was assigned to each species of frog heard at the site. A score from zero to five was given to species based on the number of nights the species was heard and the relative number of individuals calling, where a five indicated the species was heard every night and had numerous individuals calling and a zero indicated the species was never heard. The scores were used to create an overall rank for species; when a species was noted using other sampling methods (visual encounter, in refugia) but never heard, it was given a rank (lower than those species heard calling) based on the number of times it was encountered.
Blood meal identification.

Flies were homogenized under a fume hood in sterile conditions and genomic DNA was extracted using a commercial kit (DNEasy kit; Qiagen, Valencia, California, USA). Flies that had a distended abdomen but did not appear to have a blood meal were noted and were included in subsequent analyses. A polymerase chain reaction was performed on the flies using primers designed to amplify a portion of the vertebrate cytochrome B mitochondrial gene. The primers were designed to preferentially amplify cytochrome B sequences of reptile and amphibian but not culicid DNA and were validated in control experiments (Cupp et al. 2004). The sequences of the primers were 5’-CCC CTC AGA ATG ATA TTT GTC CTC A-3’ and 5’-GCH GAY ACH WVH HYH GCH TTY TCH TC-3’, where H = A, C, or T, Y = C or T, and V = A, C, or G. The PCR amplifications were carried out using reagents provided by Qiagen (Taq Master kit) in a volume of 25 µL, including 0.2 µM of each primer and ~100 ng of DNA template. Cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes, followed by 55 cycles at 94°C for 45 seconds, 50°C for 50 seconds, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The PCR amplification products were analyzed by agarose gel electrophoresis.

The products from reactions that produced a single band of expected size (~250 bp) were purified using reagents provided in a MiniElute PCR purification kit (Qiagen), and the purified products were subjected to direct DNA sequence analysis. The products that produced multiple bands, including a band of expected size, were purified using reagents provided in a QIAquick gel extraction kit (Qiagen), and the purified products were subjected to direct DNA sequence analysis. Products that did not produce a band or
produced multiple bands but not the expected bands were discarded and additional PCR amplifications were performed.

Samples not producing expected products (but known to be from flies that contained a blood meal) were amplified using alternate PCR amplifications. First, 1/10 and 1/100 dilutions of templates were made and PCR amplifications were performed according to the conditions listed above. The products from the reactions were analyzed using agarose gel electrophoresis. Second, using 1/10 dilutions of template, a “touchdown” program was used in place of the cycling conditions listed above. The cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes, followed by 5 cycles at 94°C for 45 seconds, beginning at 59°C and successively decreasing 1°C for 50 seconds, and 72°C for 1 minute, 15 cycles at 94°C for 45 seconds, beginning at 54°C and successively decreasing 0.2°C for 50 seconds, and 72°C for 1 minute, and 30 cycles at 94°C for 45 seconds, 50°C for 50 seconds, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The products from the reactions were analyzed using agarose gel electrophoresis. If either method successfully produced amplicons of predicted size, the products were purified using a MiniElute PCR purification kit (Qiagen), and the purified products were subjected to direct DNA sequence analysis.

Sequencing was performed by Clemson University Genomics Unit, Clemson, South Carolina, USA using an ABI 3730XL 96-capillary sequencer (Applied Biosystems, Foster City, California, USA) using ~100 ng template and 5 ng primer. Sequences were cleaned using Sequencher software (version 4.7, Gene Codes Corp.). Hosts were identified by 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). Known sequences that provided greater than a 95% match to searched sequences were considered positive species identifications.

Results

Collection of blood meals.

From 2001 to 2006, 356 female corethrellids were collected from resting boxes or natural resting sites. More corethrellids were collected from Tuskegee National Forest (TNF) \( n=247 \) than from Bird Pond (BP) \( n=109 \) (Table 3.1). Sampling success was higher at Bird Pond (average number of flies per box per sampling day = 0.60) than at TNF (average number of flies per box or natural site per sampling day = 0.14).

*Corethrella brakeleyi* was the most abundant species at each location \( n=172, 68.8\% \) of total at TNF; \( n=93, 85.3\% \) of total at BP) (Table 3.2). Other species collected from TNF were *C. wirthi* and *C. appendiculata*; however, only blood-fed specimens were kept and the identification of the unfed species could not be confirmed. It is known that six of the blood-fed specimens from TNF were *C. wirthi*, not *C. appendiculata*. At Bird Pond, *C. wirthi* was the only other species encountered (Table 3.2).

More blood meals were collected from Bird Pond \( n=49; 45\% \) of total at Bird Pond) than from TNF \( n=24; 10\% \) of total at TNF) (Table 3.2). Collections were evenly distributed among the nine boxes at Bird Pond, no box contained significantly more blood meals \( (G\text{-test}, X^2=6.46, d.f.=8, p= 0.60) \). At TNF, an equal number of blood-fed females were found in natural resting sites and in resting boxes \( n=5 \) each). More blood-fed female *C. brakeleyi* were collected at both locations than *C. wirthi* \( n=16 \) at TNF, 66.7% of total blood meals from TNF; \( n=41 \) at BP, 83.7% of total blood meals from BP).
Blood-fed specimens were also found in sound traps (i.e., CDC Light traps using sound as an attractant). The number of flies caught in sound traps was significantly lower than those caught from resting sites ($n=6$, 28% of blood-meals captured in sound traps at TNF; $n=18$, 37% of blood meals captured in sound traps at BP). The majority of the sound traps that captured blood fed specimens were in the Hylid Cohort 2 (see Chapter 2) ($n=13$ traps playing *Hyla cinerea*, *H. gratiosa*, or *H. squirella*; 72% of blood fed flies collected in sound traps at BP). Blood fed flies attracted to the Hylid Cohort 2 were collected on three different nights from late June to late July.

**Sampling anuran fauna.**

The Tuskegee National Forest location had 2 toad and 13 frog species ($S=15$) (Mount 1975; Cupp et al. 2004; personal observation) (Table 3.3). Bird Pond had 2 toad and 8 frog species ($S=10$) (Table 3.3). Of the anurans heard calling in large choruses, the green tree frog (*Hyla cinerea*) was encountered and heard more than any other frog at Bird Pond ($n=63$ visual encounters). The green tree frog was the only frog recorded in the refugia, usually with 2 to 3 individuals within a single refugium. *Acris gryllus*, the southern cricket frog, was ranked the second most heard and encountered species of tree frog. The eastern narrow-mouthed toad, *Gastrophryne carolinensis* (Anura: Microhylidae), was heard most nights directly after sunset, and the chorus continued calling for two hours.

The other species of frogs and toads that were heard calling at Bird Pond were only heard as individuals on certain nights or calling in groups of five or less. Some species, such as the pig frog, *Rana grylio*, and the bullfrog, *Rana catesbeiana*, called for
the entire time audio samples were taken each night; they tended to call in small groups. The other species were heard occasionally, usually as a single individual calling briefly.

**Blood meal identifications.**

Of the 73 specimens putatively determined to have a blood meal, 14 appeared to have no blood upon homogenization (19%). Of the 59 remaining specimens, 33 (10 from TNF; 23 from BP) produced amplicons of an expected size after amplification with polymerase chain reactions (Figures 3.1, 3.2). These samples were purified by gel extraction or direct purification of PCR products and sequences. The remaining 26 specimens did not produce amplicons of an expected size, although it was clear that there was DNA present after PCR (Figures 3.1, 3.2). Dilutions at 1/10 and 1/100 did not improve the PCR amplifications (Figures 3.3, 3.4); nor did the “touch down” cycling conditions (Figure 3.5).

Host species were identified from 10 flies at TNF from all four years (Table 3.4.). Only matches greater than 95% were considered correctly identified to species. *Hyla avivoca*, the bird-voiced tree frog, was identified from four flies; *H. chrysoscelis*, the southern gray tree frog, was identified from three frogs. Three flies contained DNA that matched an unknown *Rana* sp. (85% match with *Rana catesbeiana*, sequences were identical). *Corthrella wirthi* was found to feed on *H. avivoca* (*n*=2) and *H. chrysoscelis* (*n*=2); *C. brakeleyi* was found to feed on *H. avivoca* (*n*=3) and the unknown *Rana* species (*n*=3).

Host species were identified from 10 flies at Bird Pond from both years (Table 3.5). *Acris gryllus* was identified from two flies and *Rana clamitans* was identified from 8 flies. An unknown *Rana* sp. (85% match with *R. catesbeiana*) was identified from 10
flies; three sequences were degraded and were unable to be aligned. A Spearman Rank Correlation analysis (Spearman’s $r = -0.528$) indicates a negative correlation between potential host species and host species actually fed upon at Bird Pond.

**Discussion**

Comparatively, sampling was more productive at Bird Pond than at Tuskegee National Forest. It has been noted that the distributions of Nearctic *Corethrella* spp. are restricted to the Coastal Plain (<150 m above sea level) (McKeever and French 1991b). The Tuskegee National Forest study site is at a higher altitude (~30 m) than Bird Pond, this could contribute to the decreased abundance that was encountered. Although it is not known if the modifications to the collection equipment (*e.g.*, the use of fine mesh) improved trapping, future collections should use such techniques that minimize sample loss due to the small size of the flies. Resting boxes were useful in the sampling of blood fed female corethrellids as well as males and unfed and gravid females. Overall, the resting boxes worked as well as sound traps and were far more reliable (consistently contained corethrellids) in these studies.

For the first time, host species have been identified from corethrellid blood meals. The assumption that tree frogs were potential hosts of corethrellids was supported. However, it was shown that the majority of identified blood meals from Bird Pond were from a species of true frog, *Rana clamitans* (Ranidae). Although this study could not conclusively implicate other species of frogs as hosts, it is likely that the potential hosts of corethrellids include other species of frogs, including tree frogs and true frogs.

Surprisingly, host selection did not appear to be related to host abundance at Bird Pond. In fact, of the host species identified at Bird Pond, only two were from frogs that
were identified as frequently calling in choruses. The majority of hosts were identified as the bronze frog, *R. clamitans*, a species that was seldom heard. However, the bronze frog was encountered several times at night during two years of trapping (*n*=6), and was often seen during the day at the edges of the pond and near puddles at Bird Pond. It should be noted that the call of *R. clamitans* was determined to be the least preferred call of corethrellids in sound-baited traps at Bird Pond (Figure 2.7).

The Spearman’s rank correlation indicated a strong negative correlation between host abundance score and host blood meals at Bird Pond; however, only two species of frog were identified (Table 3.4). Host frogs identified at Tuskegee National Forest were different species than those at Bird Pond; the species were prominent anurans (*Hyla avivoca* and *H. chrysoscelis*) at TNF - frequently encountered and heard calling at the site (personal observation). This feeding pattern suggests the flies are locating hosts using the call, contrary to the pattern at Bird Pond.

Half of the PCR products that were sequenced failed to produce significant alignments. Reference DNA was not collected, although the complete sequence of the cytochrome B gene was available for all potential species of frog in the sequence database used. This may be due to geographic variation in the sequence of the cytochrome B gene; therefore, reference samples must be taken. Additionally, the use of reptile and amphibian primers may have limited the identification of host species; generalized vertebrate primers have been developed and must be tested on the unidentified samples.

There is still a possibility that other vertebrates are being fed upon by *Corethrella* spp. Additional attempts were made to identify the blood meals. An enzyme-linked
immunosorbent assay was performed using bird, mammal, and reptile/amphibian antisera that was produced in laboratory rabbits was used to test the identity of thirteen blood meals taken from corethrellids. Five of the corethrellid blood meals tested positive for bird, 7 tested positive for mammal, and 10 appeared to have mixed-meals. Although there were problems with the assay (e.g., sample reaction rates were much lower than positive controls), this provides further evidence to support the possibility that corethrellids feed on other vertebrates.

It is unclear why PCR failed to amplify the cytochrome B gene in many of the blood fed flies. The physiological process of blood digestion is unknown in this family; enzymes (e.g., nucleases) used in blood digestion may be degrading nucleic acids and preventing amplification. Similar studies on host feeding patterns have failed to conclusively identify host blood from some mosquito species that are suspected of feeding solely on anurans (e.g., *Uranotaenia sapphirina*) (Irby and Apperson 1988; Cupp et al. 2004).

Little is known about the mating behavior of the Corethrellidae. Polygynous mating swarms are thought to be the ancestral behavior in culicomorph flies; this is the behavior found in the Culicidae (Clements 1999). It is known that female corethrellids mate soon after emergence in laboratory colonies. It is assumed that natural populations follow a similar behavior; mating at resting sites (Yuval 2006). It was noted previously that males were collected in sound traps (Chapter 2); this conflicts with previous data (McKeever and Hartberg 1980). Blood-fed females were also attracted to sound traps. It is possible that flies are attracted to the sound of calling anurans and using the anurans as a marker to facilitate mating. Swarming near hosts is a behavior that has been
documented from species of mosquitoes (e.g., *Aedes (Stegomyia) aegypti*; Yuval 2006).

More data need to be collected about the mating behavior of the Corethrellidae.

Because there were few positive identifications of hosts, there are few inferences that can be made on host selection; for example resource partitioning may be occurring between the two sympatric species of *Corethrella* that were tested. The process of identifying the anuran hosts must be optimized, particularly for the identification of the hosts of *Corethrella* species. This would make it possible for the hosts of other species of *Corethrella* to be identified. Data such as this are essential to understanding the relationship between corethrellids and their hosts.
Table 3.1. *Corethrella* collected from resting boxes. Total number of *Corethrella* collected from resting boxes over six years and at two sites. The number of blood fed females (BM) is listed with percent of the total in parentheses. TNF = Tuskegee National Forest, Macon County, Alabama; BP = Bird Pond, Bulloch County, Georgia. * = the only specimens available from 2004 were 2 blood meals.

<table>
<thead>
<tr>
<th>Site</th>
<th>Year</th>
<th>Total</th>
<th>BM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>2001</td>
<td>45</td>
<td>6 (13)</td>
</tr>
<tr>
<td>TNF</td>
<td>2002</td>
<td>89</td>
<td>7 (8)</td>
</tr>
<tr>
<td>TNF</td>
<td>2003</td>
<td>111</td>
<td>9 (8)</td>
</tr>
<tr>
<td>TNF</td>
<td>2004</td>
<td>2</td>
<td>2 (*)</td>
</tr>
<tr>
<td>BP</td>
<td>2005</td>
<td>47</td>
<td>31 (66)</td>
</tr>
<tr>
<td>BP</td>
<td>2006</td>
<td>62</td>
<td>18 (29)</td>
</tr>
<tr>
<td><strong>SUM</strong></td>
<td><strong>356</strong></td>
<td><strong>73 (20)</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Resting box collections of *Corethrella* spp. The number of *Corethrella* spp. collected over six years at two different locations (TNF = Tuskegee National Forest, Macon Co., Alabama; BP = Bird Pond, Bulloch Co., Georgia). Total numbers are listed for each species with the number of blood-fed females (BM) listed in parentheses.

> **a** = Specimens were not available for identification; however, the numbers indicate *C. appendiculata* plus *C. wirthi* but not *C. brakeleyi*.
> **b** = Only two specimens were available from 2004, both were blood fed *C. brakeleyi*.

<table>
<thead>
<tr>
<th>Site</th>
<th>Year</th>
<th><em>C. brakeleyi</em> (BM)</th>
<th><em>C. wirthi</em> (BM)</th>
<th>C. sp.a</th>
<th>Total (BM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>2001</td>
<td>35 (2)</td>
<td>2 (2)</td>
<td>8 (2)</td>
<td>45 (6)</td>
</tr>
<tr>
<td>TNF</td>
<td>2002</td>
<td>45 (5)</td>
<td>2 (2)</td>
<td>42 (0)</td>
<td>89 (7)</td>
</tr>
<tr>
<td>TNF</td>
<td>2003</td>
<td>90 (7)</td>
<td>2 (2)</td>
<td>19 (0)</td>
<td>111 (9)</td>
</tr>
<tr>
<td>TNF</td>
<td>2004b</td>
<td>2 (2)</td>
<td></td>
<td></td>
<td>2 (2)</td>
</tr>
<tr>
<td>BP</td>
<td>2005</td>
<td>39 (26)</td>
<td>8 (5)</td>
<td>0</td>
<td>47 (31)</td>
</tr>
<tr>
<td>BP</td>
<td>2006</td>
<td>54 (15)</td>
<td>8 (3)</td>
<td>0</td>
<td>62 (18)</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>263 (57)</td>
<td>22 (14)</td>
<td>59 (2)</td>
<td>356 (73)</td>
</tr>
</tbody>
</table>
Table 3.3. Anuran species abundance at two sites. Species of frogs are listed as present or absent for a site in Tuskegee National Forest (TNF), Macon Co., Alabama and Bird Pond (BP), Bulloch Co. Georgia. Species of frogs were ranked at Bird Pond according to number of nights heard calling, size of chorus or number of individuals heard calling at a given time, and number of visual encounters. A rank of 1 indicates that *Hyla cinerea* was heard every night audio samples were taken, had large choruses, and were frequently encountered at the site. Ranks were used in calculating a Spearman’s Rank Correlation with the identification of hosts fed upon by *Corethrella* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Present/Absent at Site</th>
<th>Rank at BP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF</td>
<td>BP</td>
</tr>
<tr>
<td><em>Rana catesbeiana</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>R. clamitans</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>R. grylio</em></td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td><em>R. sphenocephala</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>Hyla avivoca</em></td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td><em>H. crucifer</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>H. cinerea</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>H. chrysoscelis</em></td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td><em>H. femoralis</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>Acris gryllus</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>Pseudacris nigrita</em></td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td><em>Bufo fowleri</em></td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td><em>Bufo terrestris</em></td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td><em>Gastrophryne carolinensis</em></td>
<td>present</td>
<td>present</td>
</tr>
</tbody>
</table>
Table 3.4. Hosts of *Corethrella* spp. based on blood-meal analysis. Hosts were identified using a PCR amplification of vertebrate-specific cytochrome B gene isolated from the midgut of blood-fed *Corethrella* spp. at two sites in the SE USA. Purified PCR products (~200 bp) were sequenced and aligned with known sequences using a database. Only alignments of > 95% match constituted a positive identification.

*a* = three specimens were identified as *Rana* sp. (85% match)

*b* = nine specimens were identified as *Rana* sp. (85% match)

<table>
<thead>
<tr>
<th>Site</th>
<th>Host species</th>
<th>Corethrella species</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF*</td>
<td><em>Hyla avivoca</em></td>
<td><em>C. brakeleyi</em></td>
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<td>TNF</td>
<td><em>H. chrysoscelis</em></td>
<td><em>C. brakeleyi</em></td>
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<td>TNF</td>
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<td><em>C. wirthi</em></td>
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<tr>
<td>BP</td>
<td><em>Acris gryllus</em></td>
<td><em>C. brakeleyi</em></td>
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Figure 3.1. Polymerase chain reactions products. A portion of vertebrate cytochrome B gene (~ 200 bp) was amplified from blood fed Corethrella spp. and visualized on a 1% agarose gel. + = products purified and sequenced, - = no product, x = products extracted from gel, purified, and sequenced, 0 = PCR conditions altered and products analyzed, MW = molecular weight markers, p = positive control.
Figure 3.2. Polymerase chain reactions products. A portion of vertebrate cytochrome B gene (~ 200 bp) was amplified from blood fed Corethrella spp. and visualized on a 1% agarose gel + = products purified and sequenced, - = no product, x = products extracted from gel, purified, and sequenced, 0 = PCR conditions altered and products analyzed, MW = molecular weight markers, p = positive control, n= negative control.
Figure 3.3. Optimization of cytochrome B PRC conditions: dilutions. A PCR amplification was performed on blood fed *Corethrella* spp to amplify the vertebrate cytochrome B gene. When analyzed on 1% agarose using gel electrophoresis, 21 specimens yielded products without expected amplicons (~200 bp). Standard dilutions were made of these and the PCR was performed again. Each pair of lanes indicates a sample: odd numbers indicate 1/10 dilution of template, even numbers indicate 1/100 dilution of template. Sixteen of these dilutions did not produce any product when analyzed on 1% agarose using gel electrophoresis. MW = molecular weight marker.
Figure 3.4. Optimization of cytochrome B PRC conditions: dilutions. A PCR amplification was performed on blood fed Corethrella spp to amplify the vertebrate cytochrome B gene. When analyzed on 1% agarose using gel electrophoresis, 21 specimens yielded products without expected amplicons (~200 bp). Standard dilutions were made of these and the PCR was performed again. Each pair of lanes indicates a sample: odd numbers indicate 1/10 dilution of template, even numbers indicate 1/100 dilution of template. Five of these dilutions did not produce any product when analyzed on 1% agarose using gel electrophoresis. p = positive, n = negative, MW = molecular weight marker.
Figure 3.5. PCR optimization: touchdown program. A PCR amplification was performed on blood fed Corethrella spp to amplify the vertebrate cytochrome B gene. When analyzed on 1% agarose using gel electrophoresis, 21 specimens yielded products without expected amplicons (~200 bp). The cycling conditions of the reaction were altered (a touchdown program) and the products were analyzed on a 1% agarose gel using electrophoresis. MW = molecular weight markers, p = positive control, n= negative control.
CHAPTER 4
CONCLUSIONS

It is clear that corethrellids prefer frequency-rich calls over simple calls and they are not locating hosts based on a similarity of the host’s call to their wing beat frequency. However, the relationship of attractive calls to identifications of hosts taken from blood fed flies found in nature does not indicate that call is a principal attractant in some systems. An intriguing pattern was found in the blood meal identifications: at Bird Pond, the majority of identified blood meals came from the Bronze frog, *Rana clamitans*. *Rana clamitans* was found to have the least preferred call at Bird Pond; whereas the species with the most preferred call, the barking tree frog, has never been identified from a blood meal. Clearly the relationship between the flies and their hosts requires further study with a primary emphasis on identifying the hosts of corethrellids in nature.

All blood meals that produced significant alignments and were identified were anuran. However, I was unable to obtain products from a PCR amplification from a large number of blood fed specimens. Because only primers used to identify reptilian and amphibian cytochrome B were used, there is still a possibility that other vertebrates are suitable hosts for corethrellids. Future analyses should use generalized vertebrate primers.

The methods used for collecting corethrellids in this study are effective and should be used in further studies. The use of resting boxes is ideal for collecting corethrellids in an unbiased manner, especially if blood-engorged flies are to be analyzed for host identification. The use of sound traps is an effective method for sampling a wide variety of species in a short amount of time; frog calls, especially the call of the barking tree frog, have shown to be effective throughout the New World and in Asia (McKeever 62
and Hartburg 1980; Borkent and Belton 2006; Bernal et al. 2006, Toma et al. 2005). However, since corethrellids show a preference for certain sounds, and there is a possibility that specific preferences differ among species, this method may not be ideal for unbiased sampling.

Further research on the hearing organs of the flies as well as sound attraction studies targeting many more species of Corethrella will benefit the study of this group. Techniques for reliably identifying blood meals should be developed. There is still much that remains unknown about the Family Corethrellidae.
WORKS CITED


Mount, R.H. 1975. The reptiles and amphibians of Alabama. Agricultural Experiment Station Publication, Auburn University, AL.


APPENDIX A

DNA SEQUENCES OBTAINED FROM BLOOD-FED FEMALE CORETHRELLIDS

A polymerase chain reaction was used to amplify a portion of vertebrate Cytochrome B gene from blood-engorged Corethrella sp. The products from the reaction were subjected to direct DNA Sequencing. The following sequences were identified to species 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).

[Format: Corethrella sp.; date collected (Day/Month/Year); collection site (BP = Bird Pond, Bulloch County, Georgia; TNF = Tuskegee National Forest, Macon County, Alabama); Species identity (BLAST match): DNA Sequence.]

Corethrella brakeleli; 15/VIII/2005; BP; Acris gryllus:

GCTATACACTACTCAGCTGATACCTTTGCTTTCATCTGTCGCCCA
TATTTGCCGAGATGTCAACAACGGCTGACTTTTACGAAATATGCATGCAA
ACGGAGCCTCATTTTTTCATCTGATATCTATATTGGTCGCGGG
CTTTATTACGGGTCTTTTCTGTTTAAAGACTTGAAACATCGGAGTTAT
TCTCTCATTATTTAGTAATAGCTACCGCATTTGCGTTTGTTGCTACCC

C. brakeleyi; 27/VII/2005; BP; A. gryllus:

GGGGNNNNNNNNNNNNNNNNNNNNNNCTTNATGGCCTTTNNTNCTGTCGCCCC
TATTTGCCGAGATGTCAACAACGGCTGACTTTTACGAAATATGCATGCAA
ACGGAGCCTCATTTTTTCATCTGATATCTATATTGGTCGCGGG
CTTTATTACGGGTCTTTTCTGTTTAAANN

69
C. wirthi; 18/IX/2001; TNF; Hyla avivoca:

CGTAGCCCATATTTTGTGAGACGTAATAACGGCTGGCTTTTACGCAATA
TTCATGCAAATGGCGCCTCATTTTTTCATCTGCATTATCTCCACATT
GGCCGAGGAATTATTACGGATCCTTTTTATTTAAAGAAACGTGAAATAT
TGGAGTTATTTCTCTCTTTTGTATAGCCACACCTTTGTGGCTATG
TCCTCCCA

C. brakeleyi; 17/VI/2003; TNF; H. avivoca:

CGTAGCCCATATTTTGTGAGACGTAATAACGGCTGGCTTTTACGCAATA
TTCATGCAAATGGCGCCTCATTTTTTCATCTGCATTATCTCCACATT
GGCCGAGGAATTATTACGGATCCTTTTTATTTAAAGAAACGTGAAATAT
TGGAGTTATTTCTCTCTTTTGTATAGCCACACCTTTGTGGCTATG
TCCTCCCA

C. brakeleyi; 2004; TNF; H. avivoca:

CGTAGCCCATATTTTGTGAGACGTAATAACGGCTGGCTTTTACGCAATA
TTCATGCAAATGGCGCCTCATTTTTTCATCTGCATTATCTCCACATT
GGCCGAGGAATTATTACGGATCCTTTTTATTTAAAGAAACGTGAAATAT
TGGAGTTATTTCTCTCTTTTGTATAGCCACACCTTTGTGGCTATG
TCCTCCCA

C. wirthi; 21/VI/2002; TNF; H. avivoca:

CGTAGCTCATATTTTGTGAGACGTAATAACGGCTGGACTTTTACGCAATA
TTCATGCAAATGGCGCCTCATTTTTTCATCTGCATTATCTCCACATT
GGCCGAGGAATTATTATGGATCCTTTTTATTTAAAGAAACGTGAAATAT
TGGAGTTATTTCTCTCTTTTGTATAGCCACACCTTTGTGGCTATG
TCCTCCCA
TGGAGTTATTCTTCTTCCTTGGTTATAGCTACAGCCTTTTGGTGTTATG
TTCTCCCA

*C. brakeleyi*; 26/IX/2005; BP; *Rana* sp.:  
CATCGCTCACATCTGCCGTGATGTAATAAACAACGGGCTGACTTTTACGCAACC
TTCATGCTAATGGCAGCATACTCTTTTTTTATCTGCATTTATTTCCACATT
GGTCGAGGCCTNTAATCTACGGATCTTTATCTCTACAAAGAAACATGGAACAT
TGGCATCATTCTCCTTTTCCTATTAATAGCCACACACCTTTTAGTCGGTTACG
TTCTCCCG

*C. brakeleyi*; 15/VIII/2005; BP; *Rana* sp.:  
GGCTGACTTTTNCGCAACCTTCATGCTAATGGCAGCATACTCTTTTTATCTGC
ATTTATTCCACATTGGCTGAGGCCTCTACTACGGATCTTTATCTCTACAAAGA
AACATGGAACATTGGCAGCATHCTCTTCCTTTTTCCNATTATAGCCACACACCTTTTG
TCGGTTACGTTCCTCCGNGAAGGACAAATATCATTTCCNAGGGG

*C. brakeleyi*; 7/VII/2006; BP; *Rana* sp.:  
CATCGCTCACATCTGCCGTGATGTAATAAACAACGGGCTGACTTTTACGCAACC
TTCATGCTAATGGCAGCATACTCTTTTTATCTGCATTTATTTCCACATT
GGTCGAGGCCTCTACTACGGATCTTTATCTCTACAAAGAAACATGGAACAT
TGGCATCATTCTCCTTTTCCTATTAATAGCCACACACCTTTTAGTCGGTTACG
TTCTCCCG

*C. brakeleyi*; 7/VII/2006; BP; *Rana* sp.:  
CATCGCTCACATCTGCCGTGATGTAATAAACAACGGGCTGACTTTTACGCAACC
TTCATGCTAATGGCAGCATACTCTTTTTATCTGCATTTATTTCCACATT
GGTCGAGGCCTCTACTACGGATCTTTATCTCTACAAAGAAACATGGAACAT
TGGCATCATTCTCCTTTTCTATTAAATAGCCACAGCTTTTGTCGGTTACGTCTCCCG

*C. brakeleyi*; 7/VII/2006; BP; *Rana* sp.:
CATCGCTCACCACATCGCGGATGATGTAAAAACACGGCCTGACTTTTACGCAACC
TTCATGCTAATGGGCGCATTCTTTCTTTTTATCTGCATTTATTTCCACATT
GGTCGAGGCGCTACTACGGATTTTCTCTTATCTCTACAAAGAAACATGGAACAT
TGGCATCATTCTCCTTTTCTATTAATAGCCACAGCTTTTGTCGGTTACGTCTCCCG

*C. brakeleyi*; 12/VII/2006; BP; *Rana* sp.:
CATCGCTCACCACATCGCGGATGATGTAAAAACACGGCCTGACTTTTACGCAACC
TTCATGCTAATGGGCGCATTCTTTCTTTATCTGCATTTATTTCCACATT
GGTCGAGGCGCTACTACGGATTTTCTCTTATCTCTACAAAGAAACATGGAACAT
TGGCATCATTCTCCTTTTCTATTAATAGCCACAGCTTTTGTCGGTTACGTCTCCCG

*C. brakeleyi*; 8/VIII/2006; BP; *Rana* sp.:
CATCGCTCACCACATCGCGGATGATGTAAAAACACGGCCTGACTTTTACGCAACC
TTCATGCTAATGGGCGCATTCTTTCTTTATCTGCATTTATTTCCACATT
GGTCGAGGCGCTACTACGGATTTTCTCTTATCTCTACAAAGAAACATGGAACAT
TGGATCATCATTCTCCTTTTCTATTAATAGCCACAGCTTTTGTCGGTTACGTCTCCCG

*C. brakeleyi*; 18/VIII/2006; BP; *Rana* sp.:
CATCGCTCACCACATCGCGGATGATGTAAAAACACGGCCTGACTTTTACGCAACC
TTCATGCTAATGGGCGCATTCTTTCTTTATCTGCATTTATTTCCACATT
GGTCGAGGCGCTACTACGGATTTTCTCTTATCTCTACAAAGAAACATGGAACAT
TGGATCATCATTCTCCTTTTCTATTAATAGCCACAGCTTTTGTCGGTTACGTCTCCCG

*C. brakeleyi*; 18/VIII/2006; BP; *Rana* sp.:
CATCGCTCACCACATCGCGGATGATGTAAAAACACGGCCTGACTTTTACGCAACC
TTCATGCTAATGGGCGCATTCTTTCTTTATCTGCATTTATTTCCACATT
GGTCGAGGCCTCTACTACGGATCTTATCTCTACAAAGAAACATGGAACATTGGCATCATTCTCCTTTTCCTATTAATAGCCACAGCTTTTGTCGGTTACGTTCTCCCG

*C. brakeleyi*; 25/VI/2005; BP; *Rana* sp.:  
ACGGCTGACTTTTACGCNANCCTTCATGCTAATGGCGCATCCTNNCTTTT  
TTATCTGCATTTATTTCCACATTTGTCGAGGCCTCTACTACGGATCTTATCTCTACAAAGAAACATGGAACATTGGCATCATTCTCCTTTTCCTACTAAN  
AGCCACAGCTTTTTGTTGTTATGTCTCCNN

*C. brakeleyi*; 11/VIII/2005; BP; *Rana* sp.:  
CNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTNTTTTACGCAACC  
TTCATGCTAATGGCGCATCCTCTTTTTTTATCTGCATTATTTCCACATT  
GGTCGAGGCCTCTACTACGGATTTATCTCTAATGCGCATCCTTTTTTTATCTCATTTATTTCCACATT  
GGTCACATTCTCCTTTTTCTATTAATAGCCACAGCTTTTGTCGGTTACGTTCTCCCG

*C. brakeleyi*; 27/VIII/2003; TNF; *Rana* sp.: >Corcytb_C44g  
ACGGCTGANTTTTACGCAACCTTCATGCTAACGGCGCATCCTTTTTTTT  
ATCTGCATCTATNTTCCACATTTGGTCGAGGCCTCTACTACGGATCTCTATC  
TCTACAAAGAAACATGGCGCATATTCTCCTATTTCTTCTCTACTAATA  
GCCACAGCTTTTTGTCGGTTATGTCTCCCA
C. brakeleyi; 22/V/2003; TNF; Rana sp.:

TNATAANGGCTGACTTCTACNAAATCTCCNTGCTAAGGAGCADCNTTNNCTTC
TTATCTGTATTTCCTCACCATCGGCAGCCCTCATTACGGGCCTATATTTA
TATAAAAGAATGNNNNNNNCNGNNNCTGNNNNNNNCNGNNNNTANCC
ACAGCTTTTGNAGGNNTGNNNNNCGNATGNNNNNNNNTNNGNNNAG

C. brakeleyi; 17/VII/2003; TNF; Rana sp.:

ACGGCTGACTTTTACGCAACCTTTGCTAAGGAGCADCCTTCTTTTTTT
ATCTGACTCTATTTCACATTGGGCTAGCCCTCATTACGGGACTCTATCT
CTACAAAAGAAACATGGAACATTGGGCTATCTTATTCCTACTAAATAG
CCACAGCTTTTGTAGGTATTGTTCCTCCCA

C. brakeleyi; 9/VIII/2005; BP; Rana clamitans:

TATCGCTCAGATCTGCGGCTATGTTAATAATGGGCTGACTTCTACGAAATC
TCGATGCATACGCGACATCTTCTCTTTTCTGTATTTACTTCCACATC
GGCCAGGCCCTCTATACGCGTACTATTTATATAAAGAAACATGAAATAT
CGGCGTAAATCTGCTATTTCTGTAATAGCCACAGCTTTTGTAGGTATG
TCCTGCCA

C. brakeleyi; 9/VIII/2005; BP; Rana clamitans:

TATCGCTCAGATCTGCGGCTATGTTAATAATGGGCTGACTTCTACGAAATC
TCGATGCATACGCGACATCTTCTCTTTTCTGTATTTACTTCCACATC
GGCCAGGCCCTCTATACGCGTACTATTTATATAAAGAAACATGAAATAT
CGGCGTAAATCTGCTATTTCTGTAATAGCCACAGCTTTTGTAGGTATG
TCCTGCCA
C. brakeleyi; 22/VIII/2005; BP; Rana clamitans:
TATCGCTCACATCTGCCGCTGATGAATTAAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACGGAGGATCATCTCTCTTTATCTGTATTACTTCCACATC
GGCCGAGGGCATTTACCGCTCATATTTATATAAAAGAAACATGAAATAT
CGGCGTAATATCGCTATTCTCTGTAATAGCCACACGCTTTTGTTGTTATG
TCCTGCAC

C. brakeleyi; 20/VII/2006; BP; Rana clamitans:
TATCGCTCACATCTGCCGCTGATGAATTAAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACGGAGGATCATCTCTCTTTATCTGTATTACTTCCACATC
GGCCGAGGGCATTTACCGCTCATATTTATATAAAAGAAACATGAAATAT
CGGCGTAATATCGCTATTCTCTGTAATAGCCACACGCTTTTGTTGTTATG
TCCTGCAC

C. brakeleyi; 12/VII/2005; BP; Rana clamitans:
TATCGCTCACATCTGCCGCTGATGAATTAAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACGGAGGATCATCTCTCTTTATCTGTATTACTTCCACATC
GGCCGAGGGCATTTACCGCTCATATTTATATAAAAGAAACATGAAATAT
CGGCGTAATATCGCTATTCTCTGTAATAGCCACACGCTTTTGTTGTTATG
TCCTGCAC
C. brakeleyi; 25/VII/2005; BP; *Rana clamitans*:
TATCGCTCACATCTGCGTGATGTATAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACCAGGCATCATTCTCTCTCTTATCTGTATTACTTCACACATC
GGCCGAGGCCTCTCTATTCGGCCTCATATTTATATAAAGAAACATGAAATAT
CGGCGTAAATCATGTATTTTCTGGTAATAGCCACAGCTTTTGTAGGTATG
TCCTGCCA

C. brakeleyi; 25/VII/2005; BP; *Rana clamitans*:
TATCGCTCACATCTGCGTGATGTATAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACCAGGCATCATTCTCTCTCTTATCTGTATTACTTCACACATC
GGCCGAGGCCTCTCTATTCGGCCTCATATTTATATAAAGAAACATGAAATAT
CGGCGTAAATCATGTATTTTCTGGTAATAGCCACAGCTTTTGTAGGTATG
TCCTGCCA

C. brakeleyi; 27/VII/2005; BP; *Rana clamitans*:
TATCGCTCACATCTGCGTGATGTATAATAATGGCTGACTTCTACGAAATC
TCCATGCTAACCAGGCATCATTCTCTCTCTTATCTGTATTACTTCACACATC
GGCCGAGGCCTCTCTATTCGGCCTCATATTTATATAAAGAAACATGAAATAT
CGGCGTAAATCATGTATTTTCTGGTAATAGCCACAGCTTTTGTAGGTATG
TCCTGCCA