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Use of acoustic signals in mating in an eavesdropping frog-biting midge

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The sensory systems of organisms are shaped by selective pressures imposed by their performance in a variety of contexts. Female frog-biting midges use the mating calls of anurans to locate their host to obtain a blood meal. Although the use of sound in foraging is well documented in this group, it is unknown whether sound is used in other contexts. To investigate the ability to use sound in nonforaging contexts, we experimentally tested the prediction that frog-biting midges (*Corethrella appendiculata*) use sound in mating. We recorded their wing beats while swarming, in controlled tethered conditions and during different- and same-sex pair interactions. Our results show sexual differences in the acoustic properties of flight tones, with male midges having higher frequency wing beats than females. Wing beats of free-flying individuals were significantly higher in frequency than those recorded from tethered individuals, cautioning the interpretation of recordings obtained following this widely used technique. In addition, interacting, tethered opposite-sex pairs altered their flight tones to match their upper harmonics, converging at the third and fourth harmonic frequencies of males and females, respectively. In male–male interactions, however, the frequency of their wing beats diverged. Therefore, flight tones in frog-biting midges may function as courtship signals attracting conspecific females and deterring rival males. We discuss the use of sound in multiple contexts in these midges and potential factors leading to their ability to eavesdrop on anurans.

Many animals depend on mating signals to attract individuals from the opposite sex. A large variety of elaborate mating displays has evolved across species, and adult males may increase the likelihood of attracting a mate by forming mating aggregations (Höglund & Alatalo, 1995). Insects are exceptional in their diverse mating strategies and often depend on sensory modalities that have traditionally received less attention (e.g. vibration: Cocroft & Rodríguez, 2005; near-field sounds: Santer & Hebets, 2008). Investigating the use of signals in such sensory modalities across different contexts provides a unique opportunity to ultimately examine the evolution of signal production and perception. In this study we investigate the acoustic mating signals used by frog-biting midges, a group in which females use sound when searching for a blood meal. Even though the use of sound in foraging in frog-biting midges is well documented (Bernal, Rand, & Ryan, 2006; McKeever, 1977; McKeever & French, 1991), the use of sound in nonforaging context has not yet been investigated.

Frog-biting midges belong to the monogeneric family Corethrellidae (Diptera) (Borkent, 2008; Borkent & Grafe, 2012). These midges are closely related to mosquitoes (Culicidae) (Stone, 1957; Wood & Borkent, 1989), and females also feed on blood to obtain protein required for egg development (McKeever, 1977; McKeever & French, 1991; Williams & Edman, 1968). In contrast to most haematophagous insects, frog-biting midges use acoustic cues to locate their host (Bernal et al., 2006; Borkent, 2008; McKeever, 1977). These midges are interspecific eavesdroppers specialized in exploiting the communication system of frogs and toads. Frog-biting midges require anuran calls to find their host and do not use other host-emitted cues commonly used by other haematophagous insects (Bernal & de Silva, in press). Given that female frog-biting midges use blood meals from anurans to support egg production, eavesdropping has critical consequences for their

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fitness. Fossil, cladistic and morphological evidence suggests frogs and frog-biting midges have been interacting probably at least since the early Cretaceous (Borkent, 2008).

Although most mosquitoes do not use sound to find their hosts (Borkent & Belton, 2006; but see Bartlett-Healy, Crans, & Gauger, 2008), the use of sound in mating is well known in Culicidae. Wing beats of mosquitoes generate air particle displacement that is detected in the near-field by conspecifics (Roth, 1948; reviewed in Clements, 1999). The use of wing beat signals in mating has been reported for a number of species across several genera of Culicidae (e.g. *Aedes*: Roth, 1948; *Anopheles*, *Culex* and *Toxorhynchites*: Downes, 1969; Clements, 1999). Both female and male mosquitoes produce courtship signals by actively modulating their flight tones to match their upper harmonics during their mating flight (Cator, Arthur, Harrington, & Hoy, 2009; Cator, Arthur, Ponlawat, & Harrington, 2011; Cator, Ng'Habi, Hoy, & Harrington, 2010; Gibson & Russell, 2005; Pennetier, Warren, Dabire, Russell, & Gibson, 2010; Warren, Gibson, & Russell, 2009). Although in mosquitoes the use of flight tones in mating has long been investigated (Roth, 1948), the role of acoustic signals in the reproductive behaviour of the closely related family of frog-biting midges has not been explored.

We recently reported that frog-biting midges form aerial mating swarms similar to mosquitoes and other closely related taxa (Clements, 1999; de Silva & Bernal, 2013). Male frog-biting midges aggregate under low-light conditions and perform characteristic mating flights. Female midges approach males in the swarm and copulation occurs in midair. In swarming species, male mosquitoes have more plumose antennae than females (Clements, 1999; Downes, 1955). The antennal hairs of males are resonantly tuned to the wing beat frequencies of female flight tones and transmit forces acting on them to the antennal flagellum, resulting in high acoustic sensitivity (Gopfert, Briegel, & Robert, 1999). Similar to mosquitoes, frog-biting midges have sexually dimorphic antennae (Felt, 1905), which suggests they also may depend on acoustic mating signals. Here we experimentally examine the wing beat frequencies and acoustic interactions among individuals of a frog-biting midge, *Corethrella appendiculata*.

To characterize the use of flight tones of frog-biting midges is necessary to examine their wing beats under natural mating conditions. In this study we investigate the flight tones of *C. appendiculata* during their mating swarms. We found differences in acoustic properties of flight tones of males and females, an indicator that this trait may be under sexual selection (Andersson, 1994). To investigate the role of flight tones in mating, we then examine acoustic interactions between same-sex and opposite-sex pairs. In particular, we study convergence on harmonically related frequencies among males and females, contrasting this behaviour with the interactions between males. We discuss the acoustic signals of frog-biting midges in relation to the wing beats of mosquitoes to provide insights about the evolution of acoustic mating signals in this group.

METHODS

Study Species

Corethrella appendiculata is a tree-hole breeding species, in which larvae are predacious and feed on aquatic insects including mosquito larvae (Lounibos, Makhni, Alto, & Kesavaraju, 2008). Adult females of *C. appendiculata* are facultative autogenous and thus do not require a blood meal to lay their first batch of eggs. We obtained *C. appendiculata* males and females from our colony maintained at Texas Tech University (Bernal Lab, Department of Biological Sciences). The colony was maintained in a $20 \times 20 \times 20$ cm mosquito-rearing cage. Details about the colony are described in de Silva and Bernal (2013). Since adult midges are sexually mature and mating takes place 4 days after emergence (de Silva & Bernal, 2013), we only used 5-day-old virgin adults to guarantee including only sexually mature individuals in our experiments.

Flight Sound Recording in Free-flying Swarms

To examine the natural flight tones of frog-biting midges, we recorded the wing beat frequencies of natural-forming swarm, which consist of over 900 male midges, while mating in their rearing cage. In general, the sex ratio is close to 1:1 in our colony. We used a particle velocity microphone (Knowles NR-21358 electric condenser microphone, Itasca, IL, U.S.A.) with an integral FET amplifier to record the flight tones of individuals in the mating swarm. This microphone offers noise cancellation and a bandwidth of 100 Hz–4 kHz. The sensitivity of the microphone is −52 dB, relative to 10 V/Pa. The microphone was placed inside the rearing cage during 1800–1900 hours, when this species is known to swarm (de Silva & Bernal, 2013). A total of 5 h were recorded during 5 days, each day with a different swarm.

Flight Sounds of Individual Midges

We recorded the sounds emitted by tethered flying male and female frog-biting midges ($N = 10$ for each sex) following Göpfert et al. (1999). We anaesthetized individuals on a custom-made CO_2 bed at 68.95 kPa (Cornelius CO_2 Gas Regulator, Cornelius Inc., Glendale Heights, IL, U.S.A.) and tethered each individual to the blunted end of an insect pin (4 cm, size 000, Bioquip 1208B, Czech Republic) using superglue (Ross Products, Inc., Columbus, OH, U.S.A.). Tethers were positioned on the dorsal surface of the abdomen, posterior to the thoracic flight muscles, allowing individuals to fly naturally. Tethered individuals were suspended 2 cm in front of a Knowles NR-21358 microphone with a custom electronic circuit used to power the microphone and amplify the recordings. The head of each midge was pointed towards the particle velocity microphone, and all sounds were recorded using a Marantz PMD660 professional sound digital recorder (sampling rate of 44.1 kHz, 16-bit resolution). Following Cator et al. (2009), we recorded the wing beat sounds of suspended midges that sustained flight for at least 10 s.

Flight Sounds of Tethered Pairs

To investigate the acoustic interactions between the sexes of frog-biting midges, we recorded the flight tones of pairs by moving a tethered flying female midge past a stationary tethered flying male following the procedure described by Cator et al. (2009). A tethered *C. appendiculata* male was positioned 1.5 cm from the particle velocity microphone and, 5 s after they initiated flight, a tethered female was introduced into the arena (10 cm). We moved the female in and out of the male's acoustic range (within 2 cm) while recording the flight tones of both individuals for 10 s before and during their interaction. We examined the acoustic interactions of 35 male–female pairs, estimating the frequency of the first five harmonics of their wing beat sounds to determine changes in flight tones during their interactions. To further examine the context in which wing beat matching occurs, we also tested the acoustic interactions between same-sex pairs. We recorded the flight tones of 10 male–male pairs by moving a tethered flying male midge past a stationary tethered flying male.