AERIAL WARFARE BETWEEN BATS AND MOTHS: EFFICACY OF ACOUSTIC APOSEMATISM, FLIGHT BEHAVIORS OF UNPALATABLE PREY, AND TWO NOVEL ANTI-BAT STRATEGIES

BY

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"If I have seen further than others, it is by standing upon the shoulders of giants."
— *Sir Isaac Newton*

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ABSTRACT

Tiger moths (Erebidae: Arctiinae) have experienced intense selective pressure from echolocating, insectivorous bats for nearly 65 million years. In response, they have evolved a suite of remarkable defenses to deal with their would-be predators. Three key innovations underlie the success of the tiger moth lineage: (1) ultrasound-sensitive ears used to hear the foraging cries of attacking bats, (2) the ability to produce ultrasound of their own, and (3) the sequestration of toxic compounds from their host plants.

In Chapter I, I provide the first field-based evidence of an acoustic aposematic function for these moth sounds. Moth sounds were effective at deterring predation attempts by free-flying bats. When the acoustic defense was removed experimentally, the moths were often captured easily, relying on their toxicity to survive. I also discovered that tiger moths vary in the degree to which they enact evasive dives in response to bat attacks. Some are nonchalant, rarely diving away from bats, while others dive much more frequently. I call this the “nonchalance continuum”.

In Chapter II, I explore how palatability underlies the difference in nonchalance between moth species. By studying the evasive flight behaviors and relative unpalatability of a suite of species, I show that more unpalatable species evade bats less frequently than less unpalatable species.

In Chapter III, I use statistical models to determine how the morphology of the sound-producing organ affects the acoustic properties of the sounds they produce. I discovered that click rate can be predicted by measuring the number of microtymbals exhibited by a species, the ratio of tymbal surface area to thorax surface area, and with

some knowledge of the phylogenetic placement of the species. These studies will aid us in understanding the evolutionary history of acoustic anti-bat defenses among tiger moths.

Finally, in Chapters IV and V, I describe two novel anti-bat defenses derived from tissues that are typically involved in sexual communication and courtship. The tiger moth *Homoeocera trizona* was found to release flocculent in response to tactile stimulation as well as when captured by a free-flying bat. The flocculent was also found to contain toxic pyrrolizidine alkaloids (PAs) as well as the male tiger moth pheromone hydroxydanaidal (HD). The tiger moth *Eucereon zizana* was found to evert their pheromone disseminating coremata in response to both tactile stimulation and bat attack echolocation. The moths and their coremata were found to contain PAs as well as HD, indicating that this behavior could be a new example of olfactory aposematism.

CHAPTER I

ACOUSTIC APOSEMATISM AND EVASIVE ACTION IN SELECT CHEMICALLY DEFENDED ARCTIINE (LEPIDOPTERA: EREBIDAE) SPECIES: NONCHALANT OR NOT?

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Research Article

Acoustic Aposematism and Evasive Action in Select Chemically Defended Arctiine (Lepidoptera: Erebidae) Species: Nonchalant or Not?

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Abstract

Tiger moths (Erebidae: Arctiinae) have experienced intense selective pressure from echolocating, insectivorous bats for over 65 million years. One outcome has been the evolution of acoustic signals that advertise the presence of toxins sequestered from the moths' larval host plants, i.e. acoustic aposematism. Little is known about the effectiveness of tiger moth anti-bat sounds in their natural environments. We used multiple infrared cameras to reconstruct bat-moth interactions in three-dimensional (3-D) space to examine how functional sound-producing organs called tymbals affect predation of two chemically defended tiger moth species: *Pygarctia roseicapitis* (Arctiini) and *Cisthene martini* (Lithosiini). *P. roseicapitis* and *C. martini* with intact tymbals were 1.8 and 1.6 times less likely to be captured by bats relative to those rendered silent. 3-D flight path and acoustic analyses indicated that bats actively avoided capturing sound-producing moths. Clicking behavior differed between the two tiger moth species, with *P. roseicapitis* responding in an earlier phase of bat attack. Evasive flight behavior in response to bat attacks was markedly different between the two tiger moth species. *P. roseicapitis* frequently paired evasive dives with aposematic sound production. *C. martini* were considerably more nonchalant and employed evasion in fewer interactions. Our results show that acoustic aposematism is effective at deterring bat predation in a natural context and that this strategy is likely to be the ancestral function of tymbal organs within the Arctiinae.

Introduction

For over 65 million years night-flying moths have been locked in an evolutionary arms race with echolocating insectivorous bats [1]. Intense selection pressure on moths has led to the evolution of ultrasound-sensitive ears that allow early detection of the sonar signals of bats and aerobatic evasion [2]. As a second line of defense, tiger moths (Lepidoptera: Erebidae: Arctiinae) evolved sound-producing tymbal organs and the ability to answer bat echolocation cries with high-intensity, broadband clicks [3, 4]. Early observations and laboratory experiments have suggested that the tymbal sounds of some species can serve an aposematic function [5–12] with the moth sounds advertising the presence of noxious chemicals sequestered in the larval stages. It has also been suggested that aposematic clicks could serve a sonar jamming or weakly jamming function [9]. Previous studies with aposematic erebids were limited because they did not record bat and moth sounds, nor did they record video to analyze flight tracks quantitatively [6, 7]. We here use both sound and 3-D videography to address whether the tymbal sounds of *Pygarctia roseicapitis* (Tribe: Arctiini) and *Cisthene martini* (Tribe: Lithosiini) function as aposematic signals vis-à-vis bats (predominantly *Myotis* species) under field conditions in Southeastern Arizona. Among moths, there are two general types of anti-bat evasive flight maneuvers: “turn away” flight and “dives” toward the ground [13]. 3-D analyses similar to those used in this study have found comparable anti-bat maneuvers in insects including preying mantids, locusts, and other non-erebid moths [14, 15, 16]. Enacting these evasive maneuvers has been measured to increase the chances of escaping predation by 40–83% depending on the species [16]. The evasive behaviors of aposematic animals have never before been quantified. It is possible that aposematic animals will infrequently

enact evasive maneuvers in response to the threat of predation, thereby exhibiting nonchalance. Alternatively, these organisms may escape predation by utilizing a diversified defensive portfolio that includes aposematic signaling in tandem with evasive maneuvering. We explore whether tiger moths produce warning sounds with or without evasive maneuvers, *i.e.* are tiger moths nonchalant or not? To our knowledge this is the first study of acoustic aposematism that allows for the 3-D reconstruction of the spatial interactions and coincidental recording of bat and moth sounds.

Results

Tymbal Sounds

We found that *Pygarctia roseicapitis* and *Cisthene martini* activate their tymbal organs both in response to the pre-recorded echolocation attack sequences of the big brown bat, *Eptesicus fuscus* and during natural encounters with free-flying bats (mostly *Myotis* species). Sounds (Fig 1) are typical of arctiine erebids in that they are composed of a series of broadband clicks produced during flexion of the tymbal (active modulation half-cycle) and during relaxation of the tymbal (passive modulation half-cycle). Our measurements agree with those previously published [17]. *P. roseicapitis* has a peak frequency of 54.0±8.4 kHz, a maximum duty-cycle of 6.1%, and produces clicks with an intensity of 76.9±4.8 dB peSPL at 5 cm. *C. martini* has a peak frequency of 60.9±7.9 kHz, a maximum duty-cycle of 5.7%, and an intensity of 74.6±7.7 dB peSPL at 5 cm. Previous work contains additional acoustic measurements for each species (See Table 1 of [17]).

Effects of Sound Production

Functional tymbals had a significant influence on the outcome of bat-moth interactions involving either *P. roseicapitis* or *C. martini*. In both species, tymbaled control (T+ group) and sham operated moths (S group) had a lower relative risk of capture than their ablated (T- group) counterparts (Fig 2). Statistical comparisons combine the "Capture, Drop" and "Consume" outcomes.

The relative risk of capture for tymbaled *P. roseicapitis* was 1.8 times less than their ablated counterparts (Fisher's exact test: $p = 0.01$, odds ratio = 7.15, 95% C.I. = [1.8, 42.0]). Bats did not capture *P. roseicapitis* with intact tymbal organs in 50% of interactions (n = 25/50) while those with ablated tymbals were not captured in only 12% (n = 3/25). No significant difference in relative risk of capture was observed between the sham-operated controls (n = 5/12) and the tymbaled group ($p = 1$). The relative risk of capture between sham-operated and ablated individuals was significant (Fisher's exact test: $p = 0.02$, odds ratio = 9.44, 95% CI = [1.5, 78.3]).

The relative risk of capture for tymbaled *C. martini* was 1.6 times less than their ablated counterparts (Fisher's exact test: $p = 0.03$, odds ratio = 11.32, 95% CI = [1.4, 527.6]). Bats did not capture *C. martini* with intact tymbal organs in 42% of interactions (n = 14/33) while those with ablated tymbals were not captured in only 6% of trials (n = 1/17). No significant difference in relative risk of capture was observed between the sham-operated controls (n = 16/30) and the tymbaled group ($p = 1$). The relative risk of capture between sham-operated and ablated individuals was significant (Fisher's exact test: $p = 0.02$, odds ratio = 13.35, 95% CI = [1.7, 626.0]).

Between *P. roseicapitis* and *C. martini* there was no difference in the relative risk of capture between tymbaled controls (p = 1), sham-operated controls (p = 1), and the ablated treatment groups (p = 1).

We examined the field recorded audio for 58 interactions which were reconstructed in 3-D and found that among interactions involving moths with intact tymbal organs (T+ and S treatments; n = 42) we were able to detect moth clicks 48% of the time (n = 20/42). We detected *P. roseicapitis* clicks in 50% of examined interactions (n = 14/28) and *C. martini* clicks in 43% (n = 6/14). This was not a significant difference in detectability between species (p = 1). We found that detecting the responses of these moths was challenging in a field setting and these percentages should be considered minimum estimates. We never detected moth clicks in interactions involving ablated moths of either species (n = 0/16).

How did moths respond to bat attacks?

To characterize how moths responded to bat cries under natural conditions we examined the inter-pulse interval (IPI) of bat cries immediately before the first detected moth clicks. The IPI is defined as the time elapsed between the onset of two sequential bat cries. IPI values for *Myotis* spp. in our recordings ranged from 5–120 ms. This analysis included only those interactions where moth clicks were detected before bat-moth minimum distance (n = 15/20) (Fig 3).

The mean IPI immediately before the first detected click was 44±3 ms for *P. roseicapitis* and 21±5 ms for *C. martini*. This difference was statistically significant (Welch t-test: p = 0.003, t = 3.91, df = 10, 95% CI = [10, 36]). This places the responses of *P. roseicapitis*

in the early approach phase and the responses of *C. martini* later, near the beginning of the late approach phase of the bat attack. There was also a trend towards a higher likelihood of "Non-Capture" outcomes in interactions with clicks generated in earlier attack phases. Interactions with *P. roseicapitis* resulted in "Non-Capture" in 100% of trials when clicks were detected in search phase (n = 2/2), but only 72% of trials where clicks were detected in early approach phase (n = 5/7). Interactions with *C. martini* resulted in "Non-Capture" in 100% of trials where clicks were detected in early approach phase (n = 2/2), but only 75% of trials where clicks were detected in late approach phase (n = 3/4).

For each interaction where clicks were detected before bat-moth contact (n = 15/20), we calculated the bat-moth distance from the 3-D data at the time of the first detected click. The mean bat-moth distance at the first detected click was 151±49 cm for *P. roseicapitis* (n = 9/15) and 43±8 cm for *C. martini* (n = 6/15). The clicks of *P. roseicapitis*' were, on average, detected at a significantly larger bat-moth distance compared to *C. martini* (Welch's t-test: p = 0.06, t = 2.14, df = 8, 95% CI = [-7, 222])).

Moth clicks rarely occurred in the theoretical 2 ms critical time window required to produce a sonar jamming effect [18, 19]. When clicking moths were avoided (n = 12/15), 86±3% of bat echoes were unaffected. When clicking moths were captured (n = 3/15), 87±6% of bat echoes were unaffected.

How did bats respond to moth clicks?

In order to assess how bats responded to moth clicks we compared bat echolocation behavior in a subset of interactions involving sound-producing tymbaled moths (T+ and

S treatments) against ablated controls for *P. roseicapitis* and *C. martini* combined (n = 19). If a bat aborts an attack sequence and returns to search phase they will produce fewer echolocation calls compared to a completed attack sequence containing a terminal buzz. Therefore, we used the number of echolocation calls produced by bats in each interaction as a measure for whether bats aborted attacks in response to moth clicks. We began counting calls once the approach phase started (defined as <50 ms IPI) and stopped counting calls once the bats returned to search phase (>50 ms IPI) (Fig 4).

We found that bats produced a significantly lower number of calls when they were hunting moths that produced clicks (MWW test: W = 84, p = 0.01). Interactions with ablated moths averaged 42±4 calls per interaction (n = 8/19) while interactions with sound-producing moths only averaged 27±7 calls (n = 11/19). In this analysis, the only interactions in the T+ and S groups which resulted in "Capture, Drop" outcomes were outliers. The single ablated moth interaction which resulted in a "Non-Capture" was interestingly the only interaction to involve a lasiuriine bat (*Lasiurus sp.*). The bat appeared to have failed capturing the moth due to the moth's evasive maneuvering (number of calls produced = 44).

Palatability

The proportion of consumed moths in the ablated treatment groups are the best representations of palatability to a bat predator because they allow palatability to be separated from the confounding effects of sound. Both *P. roseicapitis* and *C. martini* appear to be highly unpalatable. When captured, silenced *P. roseicapitis* and *C. martini* were rejected in 64% (n = 14/22) and 94% (n = 15/16) of trials, respectively (Exact binomial test: $p < 0.001$, 95% CI = [0.44, 1.0]; $p < 0.001$, 95% CI = [0.74, 1.0],

respectively). These values are similar to hand-feeding trials performed with these species using *E. fuscus* (Corcoran and Dowdy, unpublished data).

3-D Analysis of Bat-Moth Interactions

Bat flight behavior in response to sound

The minimum bat-moth distance (mBMD) is a measure of the closest distance between the bat and moth during an interaction. We compared tymbaled (T+ and S) moths that were and were not captured to ablated (T-) moths to see how bat behavior differed (Fig 5).

Among all treatments, the mBMD of captured and non-captured moths of both *P. roseicapitis* and *C. martini* were significantly different (K-S test: $p < 0.001$, $p < 0.001$, respectively). When bats did not capture the moths their mBMD was large for *P. roseicapitis* ($\bar{x}$ = 33 cm, 95% CI = [7, 146]) and smaller for *C. martini* ($\bar{x}$ = 6±1 cm).

Moth flight behavior in response to bat predation.

To determine whether *P. roseicapitis* or *C. martini* take evasive action in response to predator attacks or not, we quantified their evasive flight behaviors. We included trajectory data from 0–333 ms prior to the bat-moth minimum distance of each interaction. This time period was chosen because it encompasses the approach and buzz echolocation phases of a typical bat attack observed at our field site, during which moths are most likely to exhibit evasive behavior. This analysis includes only “Diving” behaviors as “Turn Away” flight was rarely observed.

The speed of the moths in the z-axis acts as a proxy for detecting diving behavior (Fig 6). Positive values are upward flight, values near 0 m*sec-1 are level flight, and negative values are downward flight. This calculation is similar to those employed in recent 3-D analyses of insect flight trajectories to quantitatively define diving behavior [15, 20]. A mean near 0 m*sec-1 would indicate nonchalant behavior whereas a significantly more negative mean would indicate a diversified defensive strategy that includes evasive dives.

We found that non-captured *P. roseicapitis* had a mean speed significantly more negative than 0 m*sec-1 ($\bar{x}$ = -1.01±0.35 m*sec-1). The mean z-speed of captured *P. roseicapitis* was not significantly different from 0 m*sec-1 ($\bar{x}$ = 0.19 m*sec-1, 95% CI = [-0.96, 1.83]). Both captured and non-captured *C. martini* had a mean that was not significantly different from 0 m*sec-1 ($\bar{x}$ = 0.21±0.06 m*sec-1, $\bar{x}$ = -0.03±0.11 m*sec-1; respectively).

To obtain a better estimate of the frequency of evasion among species and treatments we qualitatively examined the evasive behaviors exhibited by moths in the 58 3-D interactions as well as 36 additional interactions that were not included in our 3-D analysis (n = 94). These interactions were chosen because they had sufficient footage before and after each interaction to accurately identify the presence or absence of evasive maneuvers, only included interactions involving a single moth and a single bat, and only included interactions where a moth's evasive flight could be easily classified. Based on visual classification these interactions were scored as either "Turn Away", "Dives", or "No Evasion" as defined in classic studies of moth evasive flight [13]. Among these 94 interactions, only 7% (n = 7/94) exhibited "Turn Away" behavior. For this reason we

restricted our analysis to include only moths exhibiting “Dives” (renamed “Evasion”) or “No Evasion” (n = 87) (Fig 7).

The treatment groups (T+, T-, S) of *P. roseicapitis* and *C. martini* exhibited the same number of evasive dives (Fisher’s exact test: p = 0.84; p = 0.66, respectively). To compare the rate of evasion between species, we have pooled the treatment data for each species because treatment did not affect whether evasion was performed. *P. roseicapitis* employed evasion 2.5 times more often compared to *C. martini* (43% versus 17%; Fisher’s exact test: p = 0.02, odds ratio = 3.58, 95% CI = [1.12, 13.74]).

Discussion

This is the first study of aposematism within the bat-moth arms race in which both the bat and moth were tracked in 3-D space and acoustically recorded in nature. Our results are consistent with the idea that tymbal sounds produced by some tiger moths can act as acoustic warnings of underlying chemical defenses, *i.e.* aposematic signals. Both *P. roseicapitis* and *C. martini* respond to bat predation by making similar tymbal sounds under laboratory and field conditions. These sounds proved effective in reducing their predation by local free-flying bats (predominantly *Myotis* species). With tymbals intact, *C. martini* were 1.6 times less likely to be captured by a bat and *P. roseicapitis* were 1.8 times less likely. Bats generally kept their distance from sound-producing moths, did not enact prey capture behaviors, and increased the inter-pulse intervals of their echolocation calls after failing to produce buzz phase calls, which all suggest that they actively aborted their attacks. When moths were captured by bats they were often rejected unharmed, indicating that both moth species studied are rendered relatively unpalatable, likely by a short-range chemical-based secondary defense mediated by predator olfaction, gustation,

or a combination of the two. Our field results are consistent with earlier laboratory findings that bats can associate tymbal sounds with noxious moths and thereafter avoid them [10–12].

P. roseicapitis is a member of a clade of aposematic species that includes *Cycnia tenera* [21], a species that has been shown unequivocally to be aposematic in laboratory experiments. Both species feed on cardiac glycoside-containing plants, likely sequester similar compounds, and are unpalatable to bat predators [22–24]. The peak frequency, maximum duty-cycle, and intensity of the sounds produced by *P. roseicapitis* and *C. tenera* are similar (See Table 1 of [17]). Thus it appears that they both use aposematic strategies to deal with insectivorous bats. Likewise, *C. martini* and *Hypoprepia fucosa* (from [6, 7]) are members of a clade of erebids that are unpalatable based on their shared sequestration of secondary chemicals from lichens [25]. As above, the tymbal sounds of these two species are similar (See Table 1 of [17] and Table 2 of [26]) and appear to function as warning sounds.

It has been suggested that aposematic clicks could serve a jamming or weakly jamming function [9]. Most research suggests that jamming occurs via ranging interference [18, 19, 27, 28]. The duty-cycles of the sounds produced by *P. roseicapitis* and *C. martini* are much lower than those of *Bertholdia trigona* [17], the only proven sonar jammer (~6% versus 44%) and because of this we argue that it is unlikely that these sounds produce a strong jamming effect. In interactions with *P. roseicapitis* and *C. martini* over 85% of bat echoes were unaffected by moth clicks, suggesting a weak jamming effect at best. This further supports an aposematic function for the sounds produced by *P. roseicapitis* and *C. martini*.

P. roseicapitis and *C. martini* produced sounds in response to inter-pulse intervals associated with the approach phase of bat echolocation. Studies with the dogbane tiger moth, *C. tenera* showed they responded most often to a pulse repetition rate of 30–50 calls*sec-1 which is equivalent to an inter-pulse interval of 20–33 ms [29]. Our results include responses within the range reported for *C. tenera*. However, the mean IPI that *P. roseicapitis* responded to was much longer while *C. martini* was near the lower limit of this range. A more recent study that examined the timing of responses within a diverse group of neotropical tiger moths showed that species can vary drastically in the timing of their responses [30]. The difference in timing of clicks between *P. roseicapitis* and *C. martini* is therefore not unexpected and both species' first responses fall within the range reported for other tiger moths. It is unknown what factors might contribute to variation in the timing of acoustic responses and without a well-supported phylogeny analyzing this data while taking into account shared ancestry is impossible.

The bat-moth distance when *P. roseicapitis* and *C. martini* clicks were first detected were somewhat smaller than those reported for *B. trigona* (See Fig 6A of [20]). These species are much quieter and produce fewer clicks per unit time compared to *B. trigona* so it is possible that this could be explained by a bias towards later detection times in this study. In interactions where moths were detected to click the earliest, we did not observe major changes in bat trajectories or "turn aways" as has been described in other 3-D field studies in response to sonar jamming signals [20]. Instead, the bats in this study typically made relatively minor adjustments to their trajectories, but did not make capture attempts. This behavior has been recorded in lab conditions with *C. tenera* against the bat *E. fuscus* (Hristov and Conner, unpublished). *P. roseicapitis* produced clicks farther away and

ended interactions farther from bats than *C. martini*. It is possible that this difference in minimum bat-moth distance could be attributed to *P. roseicapitis* clicking earlier, the greater use of evasion by *P. roseicapitis*, or a combination of the two.

We were surprised by how often both species were captured by the bats and then rejected compared to previous laboratory and field data where most were not captured [6, 7, 10, 11]. There are several possible explanations. Despite the fact that most of the moths were rejected by bats, the level of chemical defenses may not be high or they may be variable among individuals, *i.e.* automimicry. Another possibility is that the area, which is particularly speciose in moth fauna, may carry a high load of Batesian mimics. Either scenario may make a sample-and-reject strategy viable [31]. It is also possible that the bat population was dominated by inexperienced young of the year. These animals undoubtedly require time to learn the aposematic signal and to associate it with noxious prey. The bat-moth season in the Chiricahua Mountains is tied to the local monsoons and is particularly compressed in time (~4–6 weeks total per year). This may also be a contributing factor in that competition for food at this time of the year is particularly intense. This phenomenon could also be partly due to tymbaled moths failing to respond to bat echolocation for unknown reasons. We currently do not have sufficient data to test these possibilities.

It is possible that *C. martini* and *P. roseicapitis* are part of a larger acoustic Batesian, quasi-Batesian, and/or Müllerian mimicry ring(s) in Southeastern Arizona. We are in the process of collecting data on additional moth species in the area. The target taxa include *Carales arizonensis*, *Pygarctia murina*, and *Ctenucha venosa*.

In the only other field studies of acoustic aposematism, the moths, *H. fucosa* (Lithosiini) and *Halysidota tessellaris* (Arctiini) were said to fly straight with no obvious evasive maneuvers [6, 7]. In contrast, the responses of *P. roseicapitis* and *C. martini* to bat attacks are more varied and include aposematic signaling and, in some cases, evasive maneuvers like dives. The 3-D flight tracks and behavioral scoring show that nearly half of *P. roseicapitis* produce aposematic clicks in tandem with evasive dives. In comparison, *C. martini* can be considered nonchalant, diving in only 17% of interactions. This suggests that, with respect to evasive responses, species can lie in different places along a nonchalance continuum.

In those early field studies of aposematic erebids neither quantitative measurements of bat-moth flight trajectories nor the acoustic responses of moths to foraging bats were included. By recording and filming interactions between aposematic tiger moths and bats under natural conditions we have presented the first quantitative data detailing (1) how sound production influences the outcome of these interactions, (2) how these moths and bats alter their flight behaviors during these predation events, (3) how these moths time their acoustic signals, and (4) how bats change their echolocation in response. These results highlight the strengths of a quantitative, comparative approach in understanding the diversity of strategies within the bat-moth arms race. All aposematic tiger moths do not respond to bat predation the same.

Based on the most recent phylogenetic analysis of the subfamily Arctiinae (Family: Erebidae), *P. roseicapitis* and *C. martini* are members of two separate, monophyletic tribes (Arctiini and Lithosiini, respectively) [21]. The primary difference between these tribes is their larval feeding behavior. Arctiini feed on a variety of plants including those

containing pyrrolizidine alkaloids and cardenolides [21] whereas members of Lithosiini feed as larvae on lichens and are known to sequester polyphenolic defenses, likely from the algal symbiont in the lichen [25]. Phylogenetically, the Lithosiini are positioned basal to the Arctiini. Our data suggest that acoustic aposematism may be a synapomorphic character for all members of the subfamily Arctiinae. This hypothesis should be tested with rigorous phylogenetic methods.

To fully understand the evolutionary history of the bat-moth arms race we need to examine the variety of anti-bat defenses deployed by arctiines on a larger scale and within a complete phylogenetic framework. This would add much needed resolution to the picture of how this predator-prey system has come to exist in its present form.

Methods and Materials

Ethics Statement

No vertebrates (bats) were captured or handled during these experiments. All data involves free-flying bats in their natural habitats. No state or federal permits were required to conduct this work. The methods of this study were approved by the Wake Forest University Institutional Animal Care and Use Committee (protocol #A12-048). This work was performed with permission on private property.

Field Site

Field experiments were conducted at the Southwestern Research Station (SWRS) operated by the American Museum of Natural History. SWRS is located in Cochise County approximately 7 km southwest of Portal, Arizona, United States. The GPS coordinates of the field site are: 31°53’00.30” N 109°12’27.20” W; elevation: 1,650 m.

This site was chosen for its high diversity of both bats and moths. The field trials were performed between July 18th and August 10th during 2011, 2012, and 2013.

Moth Collection and Manipulation

Moths were collected on station grounds from sheets illuminated with 15 Watt ultraviolet "quantum" lights (Leptraps.com; F15T8QBL). Moths identified as either *Pygarctia roseicapitis* or *Cisthene martini* were stored individually for up to 24 hours in 30mL plastic containers at ambient temperatures. These species were targeted because they have previously been shown to produce sound in response to bat echolocation [17] and are thought to sequester defensive compounds from their larval hosts. Larval *P. roseicapitis* are known to feed on toxic *Euphorbia* species [23], and at SWRS we found them feeding on Desert Milkweed (Asclepias angustifolia). They can be reared on other cardiac glycoside-containing plants including *Apocynum cannibinum* (Dowdy, pers. obs.). Species of *Cisthene* and other Lithosiines have been reared on lichens which can contain polyphenolic compounds and those compounds have been found in the tissues of adults [25, 32, 33]. *P. roseicapitis* (Arctiini) has a forewing length of 1.4–1.7 cm and both fore- and hindwings are pearly white with a contrasting red head and abdomen. *C. martini* (Lithosiini) has a forewing length of 0.9–1.1 cm with orange and black coloration on the forewings, red coloration on the hindwings, and a red or orange abdomen (Fig 1).

Individuals were randomly placed into one of three treatment groups: Tymbals Intact (T+), Tymbals Removed (T-), and Sham Control (S). Moths from all three treatments were placed in individual vials and chilled for 5 minutes in an ice bath prior to surgery. The T+ group was removed from the ice bath and no further manipulations were performed. In the T- group tymbal organs were ablated with curved forceps by removing

the cuticular surface of the organ. The space below the tymbal organ's surface is a small, air-filled chamber, so this ablation did not cause any discernable injury or loss of haemolymph. Loss of tymbal function for this group was verified by manipulating the individual while monitoring for sound production with an ultrasonic detector (Pettersson Model D-100). In the S group the scales surrounding the areas of the tymbal organs were removed using curved forceps to simulate experimental manipulation without removal of the sound-producing organs. Individuals in all treatment groups were then assigned random ID numbers as designators such that their group assignment was not known to experimenters during field releases and data analysis. We cross-referenced ID numbers after data analysis was complete to match data with their respective treatment and species identities.

Outdoor Flight Arena

Two ultraviolet lights were placed approximately 5 m off the ground and set 4 m apart in the center of an open grassy field. This area is approximately 600 m2 and surrounded primarily by Arizona sycamore (*Platanus wrightii*), scrub oak (*Quercus turbinella*), and alligator juniper (*Juniperus deppeana*). The ultraviolet lights served to increase general insect abundance at the field release site as well as to provide low-intensity ambient light to the area. The insects drew in free-flying bats which began to forage reliably in this outdoor flight arena. Moths included in this study were released at this site after treatment, one at a time, starting after sunset (21:00) for six hours (03:00) or until we ran out of moths to run in trials. The majority of releases involved the moths taking off shortly after being released from their containers. In a few cases we released the moths by tossing them up in the air. In these cases, we did not collect data for 5–10 seconds or until

it was clear the moth was flying under its own power and would be able to react normally to any bat attacks. This was confirmed using the video recordings. We recovered any experimental moths that were not captured or that did not fly away in order to keep the number of experimental moths flying at one time to a minimum. There was almost always only one experimental moth flying at a time.

Audio Recording and Analysis

Audio of the bat-moth interactions was recorded using three Avisoft Bioacoustics CM16/CMPA ultrasonic microphones (Berlin, Germany) with an Avisoft Ultrasound Gate 416H recording interface. Two microphones were placed approximately 1.5 m high and 4 m apart, near the edges of the recording volume, pointed up towards the interaction space. These stationary microphones aided in registering echolocation calls to bats filmed within the flight arena. The third microphone was mounted on a pole wielded by a central observer and maximized the likelihood of detecting moth sounds by minimizing the distance between the microphone and the moth. The pole was held approximately 1–3 m from the moth during field releases. All recordings were analyzed in Avisoft SASLab Pro v5.2.

Inter-pulse intervals (IPI) of bat calls for each interaction were determined by calculating the time elapsed between the two bat calls immediately preceding the first detected moth clicks. We have defined our bat attack phases in terms of IPI as: <5–7 ms (buzz), 8–20 ms (late approach), 21–49 ms (early approach), >50 ms (search). To convert IPI to pulse repetition rate we calculated and reported the number of calls (pulses) that would occur in 1 second at a given IPI. The time of the first detected moth click was cross-referenced

with our 3-D flight path data to determine the bat-moth distance when the moth first clicked.

To determine whether moth clicks had the potential to jam bat echolocation we measured the frequency of their occurrence within the 2 ms critical window of each bat echo for the interactions reconstructed in 3-D [18, 19]. We used only echoes of bat calls that were produced after the search phase ended (<50 ms IPI) and before the next search phase began (>50 ms IPI) for each interaction. This allows for a standardized comparison to be made between interactions which vary in the number of bat calls they contain. For each bat call we used the middle of the call to represent the time point at which the call was emitted. This time point was cross-referenced with our 3-D flight path data to determine the bat-moth distance at that time. We then calculated when the echo of that call would return to the bat by assuming a speed of sound of 343 m*sec-1 and a travel distance of twice the bat-moth distance (time to travel to the moth and back). The critical window was calculated to be the echo's return time point ±2 ms. We then calculated when the moth clicks would arrive at the bat. If any of the moth clicks occurred within the critical window we scored this as having the potential to jam that bat echo. To determine the number of unaffected bat echoes in each interaction we took the number of critical windows which were not overlapped by moth clicks and divided by the total number of bat echoes used in the critical window assessment.

Video Recording

Videos of the interactions were recorded using three Basler AG Scout infrared cameras (Model scA640-120gc; Ahrensburg, Germany) at 60 frames*sec-1 with 640x480 resolution. The cameras were synchronized with the audio recordings using custom

hardware (Innovision Systems, Columbiaville, MI, USA). Video was acquired with MaxTraq2D software (Innovision Systems) and two Intel PRO/1000 PT Dual Server Adapters (Intel, Model: EXPI9402PT) installed in a PC running Windows 7. Six Wildlife Engineering IR-Lamp6 lights (Tucson, AZ, USA), two Bosch UFLED20-8BD illuminators (Farmington Hills, MI, USA) and two Raytec Raymax 200 platinum illuminators (Ashington, UK) provided infrared illumination in the flight arena.

3-D Reconstruction

The relative orientation method was used to calibrate the cameras to perform three-dimensional (3-D) reconstruction of bat and moth trajectories [34]. These methods were applied in MaxTraq3D software (Dynamic Wand Method; Innovision Systems). Two spherical, infrared-reflecting markers were fixed a known distance apart on a T-shaped calibration wand. The calibration wand was rotated and translated throughout a subset of the flight arena's volume, recorded, and digitized. An L-shaped calibration frame was also constructed to set the origin of the 3-D coordinate system. The calibration frame has four points set at a known distance from each other and is held motionless in one location within the recording volume during calibration. This configuration allowed for a calibration volume of approximately 90 m3 (4 m x 5 m x 4.5 m) with a maximum spatial error of 3.7 cm.

A visual observer tracked each released moth while that moth flew in the calibrated volume of the flight arena. An interaction was recorded if the visual observer within the calibrated volume (<1–2 m from moth) judged that a bat was flying near to the moth of interest and, in some cases, heard the bat's terminal buzz calls. Further screening was performed on recorded interactions after filming to ensure that bats were flying towards

released moths rather than flying past them. We found that most bats that were not interested in the released moth were either clearly interested in a different insect within the flight arena or flew much higher in an airspace that was not included in our recorded calibrated volume. We included only interactions involving one bat and one moth in our data analysis.

We filmed 167 bat-moth interactions with easily discernable outcomes. For 3-D analyses we used a subset of 58 interactions with the highest quality and lowest spatial errors. These bat-moth flight trajectories were digitized in MaxTraq2D. The locations of individuals were represented as single point centroids determined by a center of mass calculation. The digitized frames representing bat and moth locations through time were imported into MaxTraq3D for further conversion into 3-D (x, y, z) coordinates readable by MATLAB (Natick, MA, USA). A custom MATLAB script (BATracker; coded by Brad Chadwell) and a smoothing spline function (MATLAB spaps routine) were used to generate and smooth each flight path. Smoothed flight paths were then used to estimate flight parameters.

Behavioral Scoring

Experimental moths were individually selected at random from the pool of available individuals. Moths were pre-warmed with a heat lamp to ~27°C to insure maximum flight performance and released from the center of the flight arena and tracked visually and recorded (video and audio).

Bat-moth interaction outcomes were separated into 3 categories (Fig 8). (1) Non-Capture: The bat turned toward the moth and closed in distance, but did not make contact with the

moth. (2) Capture, Drop: The bat captured the moth in the tail or wing membrane and then released it. (3) Consume: The bat captured the moth in the tail or wing membrane and it was not released; assumed to have been consumed.

Bat Species Identification

Automated acoustic species recognition software was utilized to identify the bat species foraging during data collection (Kaleidoscope Pro 2.0, Wildlife Acoustics, Inc., Concord, MA). The species classifiers for the Arizona region and the "+1 More Accurate" setting was used during classification. In addition, we verified these identifications by hand using call structure and frequency content. Of the 167 interactions in this study, 85% were determined to belong to the genus *Myotis*, 3% were lasiurine, 1% were *Eptesicus fuscus*, and 10% were unable to be confidently classified. The 58 digitized interactions were also predominantly (>85%) comprised of bats in the genus *Myotis*. Nearly all bats included in the analysis of the timing of moth clicks (Fig 3) and the number of bat echolocation calls produced between search phases (Fig 4) were verified to include *Myotis* exclusively. The only exception was a single data point from an ablated moth which was determined to involve a lasiurine.

Lab Recording of Moth Sounds

Freshly captured moths were held by the wings folded above the thorax using a hemostat. All recordings were made in a darkened room. An Avisoft Bioacoustics USGH digital recording unit was connected to a single Avisoft CM16/CMPA ultrasonic microphone (± 3 dB from 15–140 kHz) and set to record at a sampling rate of 250 kHz. The microphone was placed perpendicular to the midline of the moth body, 10 cm from the thorax of the

individual (where sound-producing organs are located). An AT 100 ultrasonic speaker (Binary Acoustic Technology) was placed 10 cm from the posterior end of the moth thorax (where tympanal hearing organs are located), parallel to the midline of the body. Moths were stimulated to produce sound by playing a pre-recorded echolocation attack sequence from the sympatric insectivorous bat, *E. fuscus*. Search, approach, and buzz phases of bat echolocation were all present and spanned a pulse interval of 115 ms in search phase to 6 ms in the buzz phase. Echolocation intensity reached and then sustained a peak equivalent Sound Pressure Level of 100 dB at 10 cm in the approach phase. For more details see previously reported methods [30]. Stimuli were repeated seven times per individual with approximately 4–5 seconds of silence between trials.

Statistics

Statistical analyses of observation data as well as 3-D data were performed in R version 3.2.0 [35].

Means are reported with the standard errors on the mean. If distributions violated the assumption of normality the data were log10 transformed in order to fit a normal distribution as confirmed by the Shapiro-Wilk test and 95% confidence intervals are reported instead of standard errors on the mean. Fisher's exact test was used to test for independence between the three nominal treatment variables (T+, T-, S) and two nominal outcomes (“Non-Capture”, “Capture, Drop” combined with “Consume”). Fisher’s exact test was also used to test for differences in the frequency of evasion among treatments within species and between the two species.

Palatability was assessed using the Exact Binomial Test. Rejections by bats were coded as "successful" trials, the hypothesized null probability of rejection was 0% (perfectly palatable), and the alternative hypothesis was that the true probability of rejection was greater than 0%. Welch's t-test was used to test for differences in the mean inter-pulse interval as well as the mean minimum bat-moth distance between species. The nonparametric Mann-Whitney-Wilcoxon test (MWW test) was used to test for a significant difference in the number of calls produce by bats attacking sound-producing tymbaled versus ablated moths. When testing for differences in some 3-D trajectory analyses the assumption of normality was violated. In these cases we used the non-parametric Kolmogorov-Smirnov test, referred to as the K-S test in this text.

Tests for unequal variances were performed using Levene's Test implemented in the lawstat package for R after removal of outliers [36]. Outliers were chosen by log10-transforming and removing values more than 1.5*IQR (inter-quartile range) beyond the 75th percentile.

P-values were adjusted using the Bonferroni Correction method when performing multiple comparisons. Adjusted p-values greater than 1 are reported as 1. The standard alpha of 0.05 was used. To control for the possible effects of pseudoreplication we analyzed only the first interaction of each individual moth.

The number of individual bats frequenting the calibrated space at any given time varied from one to six. This resulted in some unavoidable pseudoreplication across individuals. This study covered a period of three years which should increase the probability of involving unique bats. We consider this an inevitable drawback of working with free-flying individuals.

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Fig 1. Morphology and acoustic emissions of *Pygarctia roseicapitis* (A-F) and *Cisthene martini* (G-L). (Continued on Next Page).

The moths (**A, G**) and their corresponding tymbal organs (**B, H**), oscillogram (**C, I**), spectrogram (**D, J**), power spectral density plot (**E, K**), and the spectrogram of their response to simulated bat cries (**F, L**) are shown. Tymbal images are oriented with anterior on the left and ventral on the top with some scales removed. Insets show the relative position, orientation, and size of the tymbal (yellow) organ and microtymbals (red) on the thorax of each species. Insets are oriented with anterior on the left and dorsal on the top. Oscillogram, spectrogram, and power spectral density plots (**C-E, I-K**) show a single activation and relaxation (modulation cycle) of the tymbal organ. Moth responses to simulated bat cries (**F, L**) show each species' earliest response and do not correspond to the same segment of time. Bat cries are brightest and sweep from higher to lower frequencies within a single call. Moth clicks are broadband and cluster in groups of clicks.

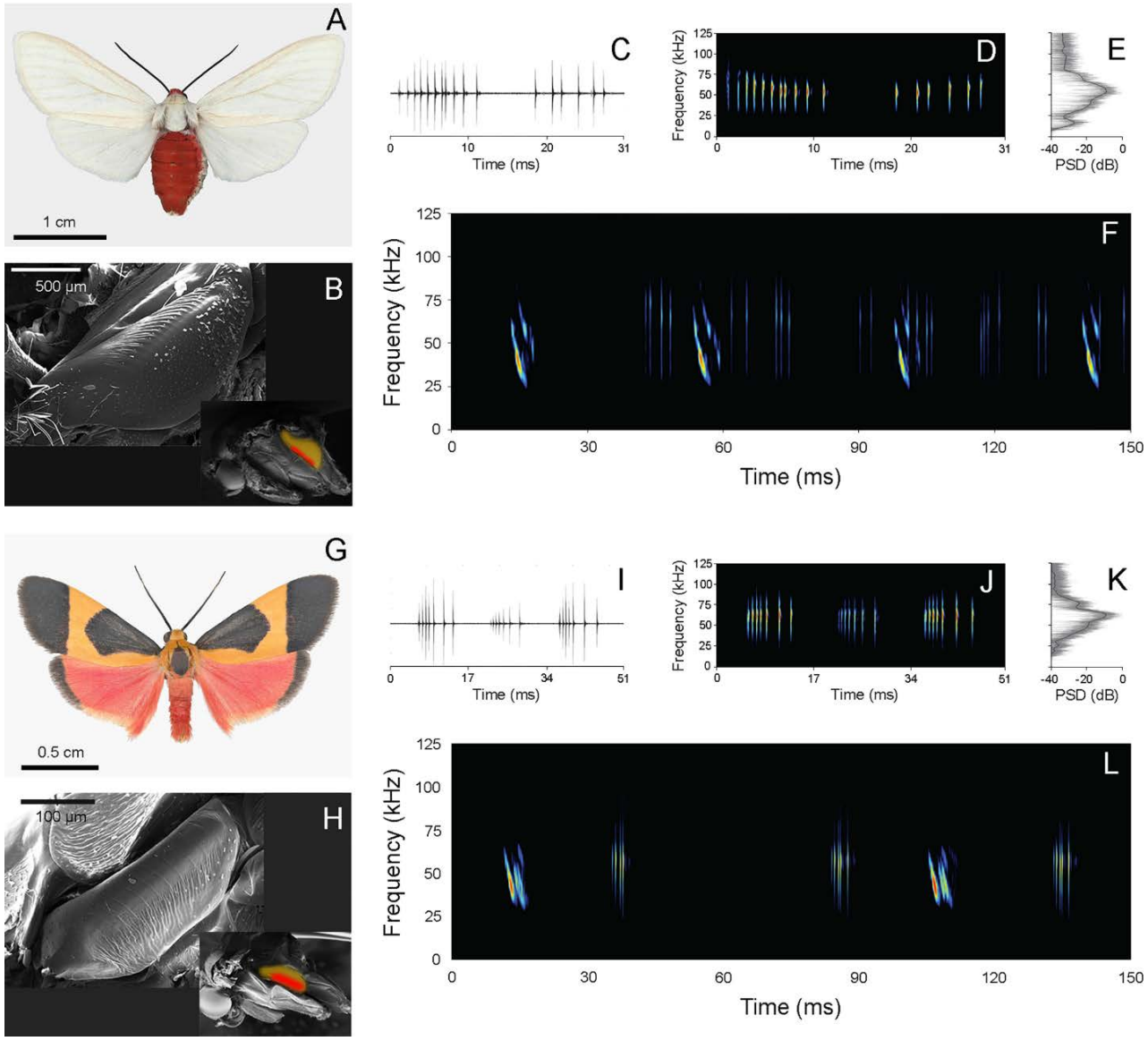

A
1 cm
B
500 µm
C
Time (ms)
D
Frequency (kHz)
Time (ms)
E
PSD (dB)
F
Frequency (kHz)
Time (ms)
G
0.5 cm
H
100 µm
I
Time (ms)
J
Frequency (kHz)
Time (ms)
K
PSD (dB)
L
Frequency (kHz)
Time (ms)

Fig 2. Effect of functional tymbals on the outcomes of bat-moth interactions for *Pygarctia roseicapitis* and *Cisthene martini* (Continued on Next Page).

The percentages of interactions for each possible outcome recorded for each treatment group. Numbers within each bar indicate the number of interactions observed for that treatment/outcome combination.

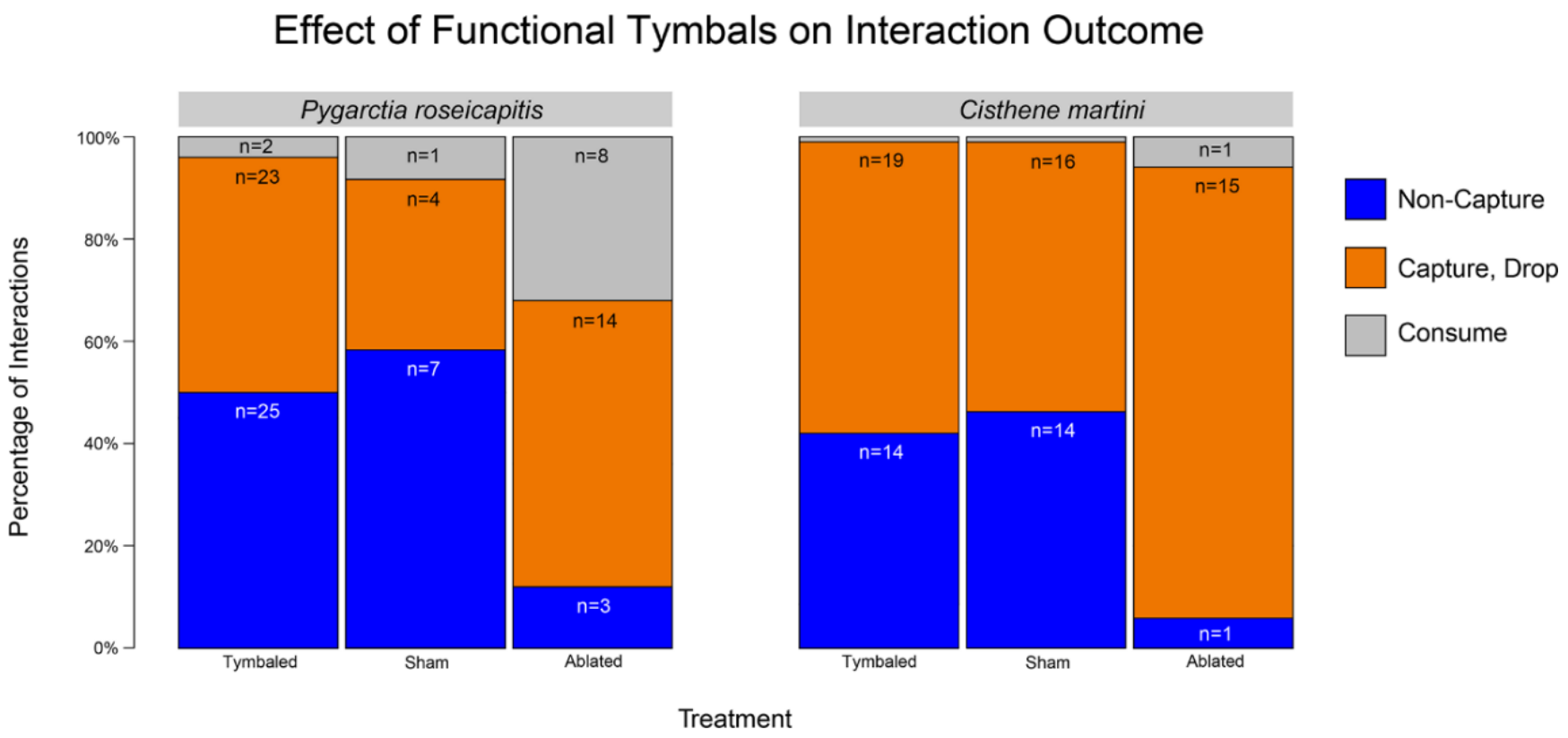

Effect of Functional Tymbals on Interaction Outcome
Pygarctia roseicapitis
Cisthene martini
Percentage of Interactions
100%
80%
60%
40%
20%
0%
n=2
n=23
n=25
n=1
n=4
n=7
n=8
n=14
n=3
n=19
n=14
n=16
n=14
n=1
n=15
n=1
Tymbaled
Sham
Ablated
Tymbaled
Sham
Ablated
Treatment
Non-Capture
Capture, Drop
Consume

Fig 3. Inter-pulse interval (IPI) between the two bat calls immediately preceding the first detected moth clicks for "Tymbaled" (T+ and S groups), sound-producing *Pygarctia roseicapitis* and *Cisthene martini*. (Continued on Next Page).

Box plot upper and lower hinges represent the 25th and 75th percentiles of their respective distributions. The 50th percentile (median) is shown as a thicker black line between hinges. Tukey-style whiskers extend from each hinge to the most extreme value within 1.5*IQR (inter-quartile range). Actual data from which the box plots are constructed are displayed as points jittered along the midline of their respective box plot. Any data points beyond the whiskers are outliers. "Non-Capture" outcomes are colored black and "Capture" outcomes are colored red. Bat attack phases and their corresponding range of IPI's are indicated as: Search Phase (white), Early Approach (light grey), Late Approach (dark grey), and Buzz (black). The right y-axis are the values of pulse repetition rate (pulse*sec^{-1}) corresponding to the values of IPI.

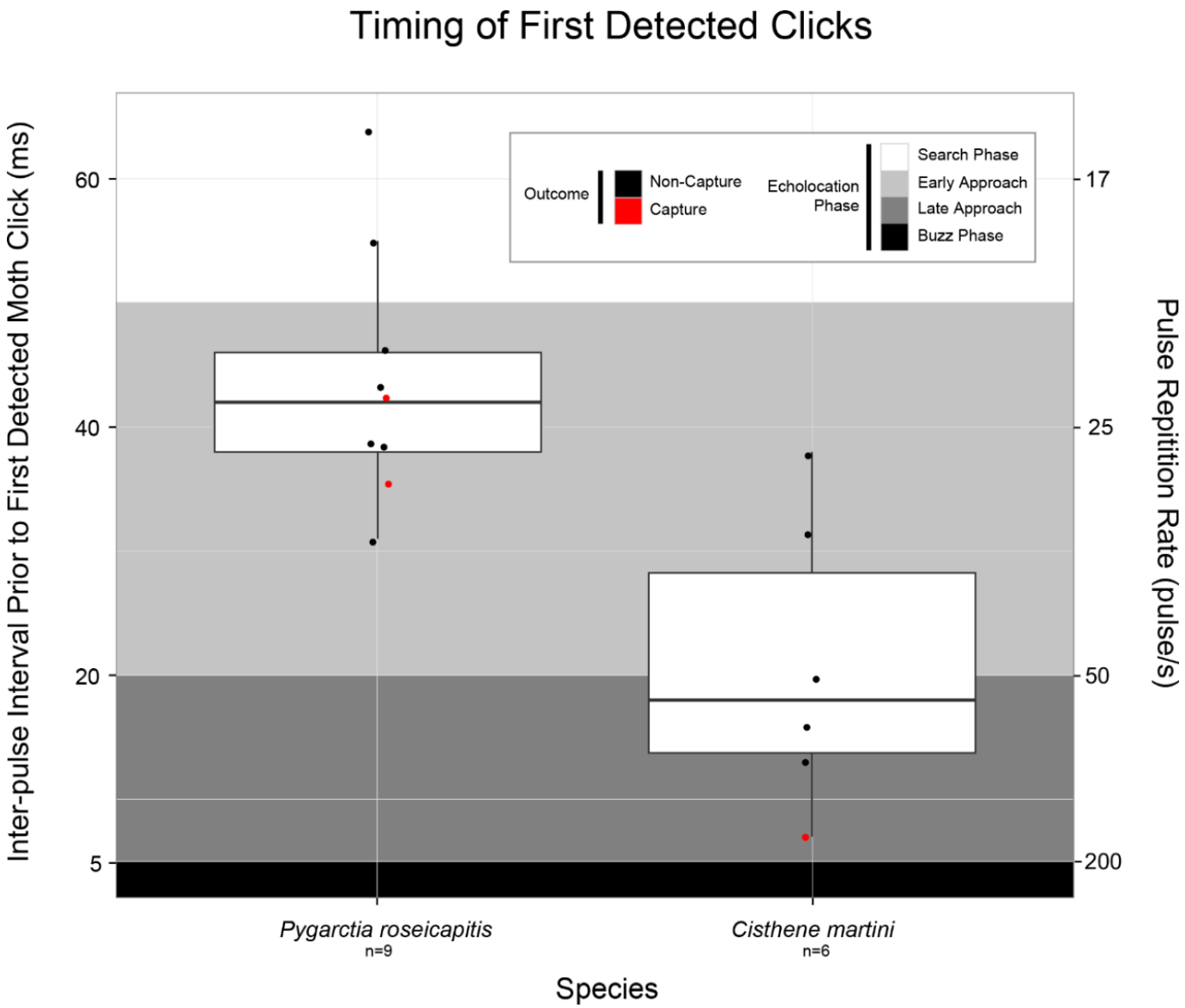

Timing of First Detected Clicks
Inter-pulse Interval Prior to First Detected Moth Click (ms)
Pulse Repitition Rate (pulse/s)
60
40
20
5
17
25
50
200
Outcome
Non-Capture
Capture
Echolocation Phase
Search Phase
Early Approach
Late Approach
Buzz Phase
Pygarctia roseicapitis
n=9
Cisthene martini
n=6
Species

Fig 4. Number of echolocation calls bats produced between search phases for "Tymbaled" (T+ and S groups), sound-producing *Pygarctia roseicapitis* and *Cisthene martini*.

Boxplot follows plotting conventions in Fig 3. For each interaction, moth species identity has been coded as shape and the outcome of each interaction is coded by color.

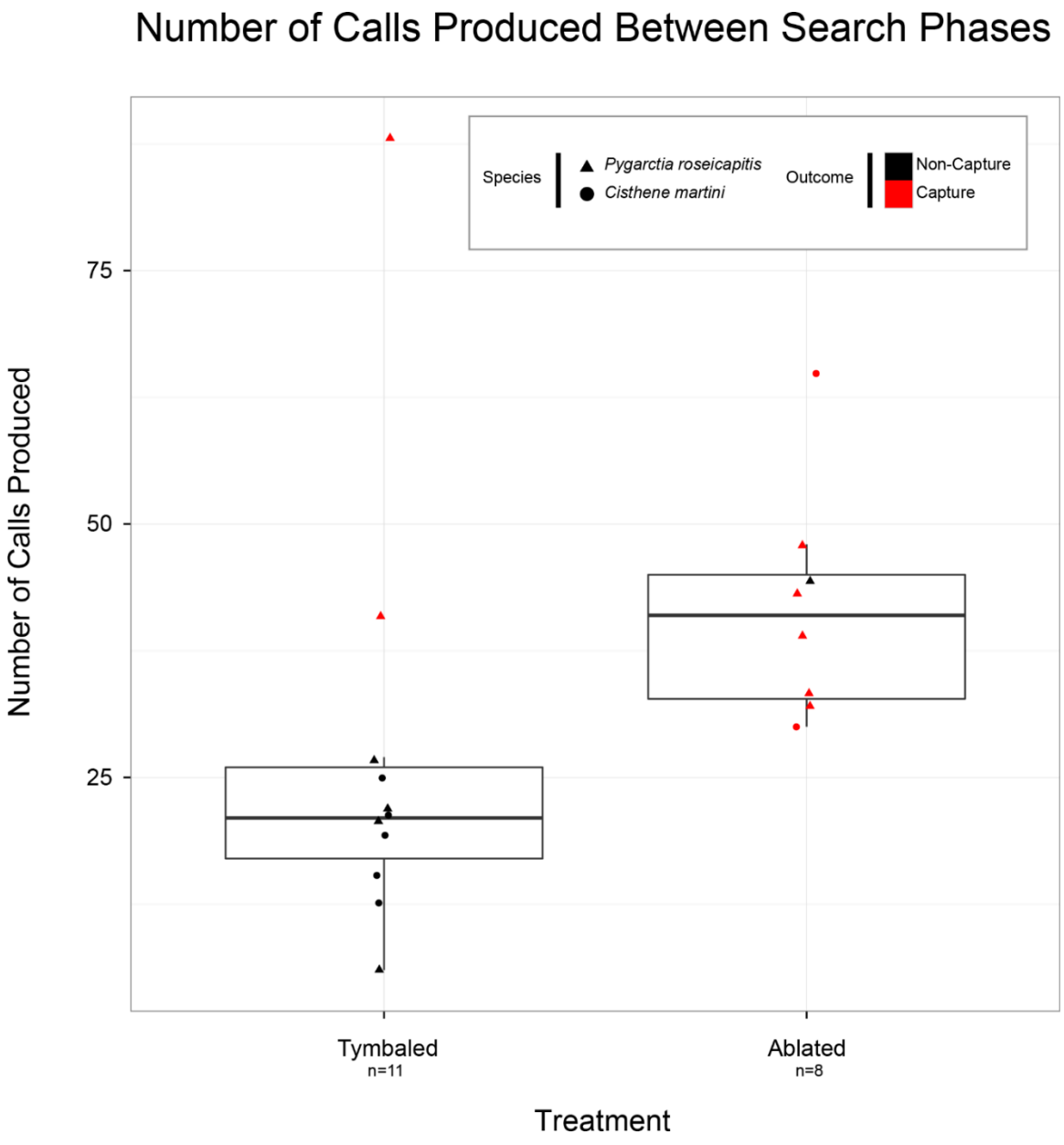


Fig 5. Minimum bat-moth distances (mBMD) between "Capture, Drop" and "Non-Captured" outcomes among "Tymbaled" (T+ and S groups) and "Ablated" (T-) moths for *Pygarctia roseicapitis* and *Cisthene martini*. (Continued on Next Page).

Boxplot follows plotting conventions in Fig 3. Horizontal dot-dashed line demarcates 3.7 cm which was the most conservative of the smallest minimum bat-moth distances in which we could measure due to inherent limitations and error in the 3-D reconstruction process. Bat and moth should be considered to be occupying the same coordinates below this value. This plot displays the closest distance between bats and moths (T+ and S treatments) during each interaction. Interactions that resulted in "Capture, Drop" were all below 3.7 cm. All interactions that resulted in "Non-Capture" were above 3.7 cm.

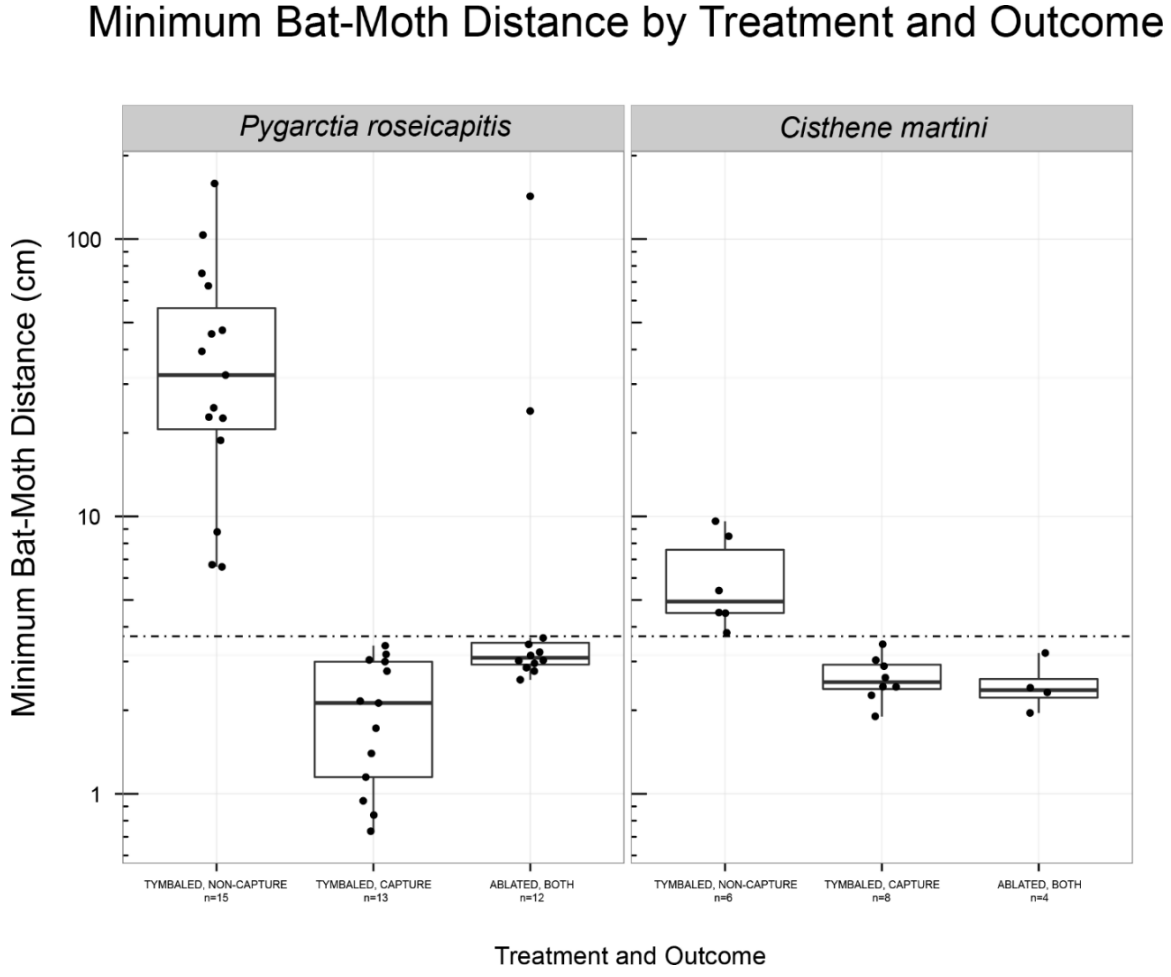
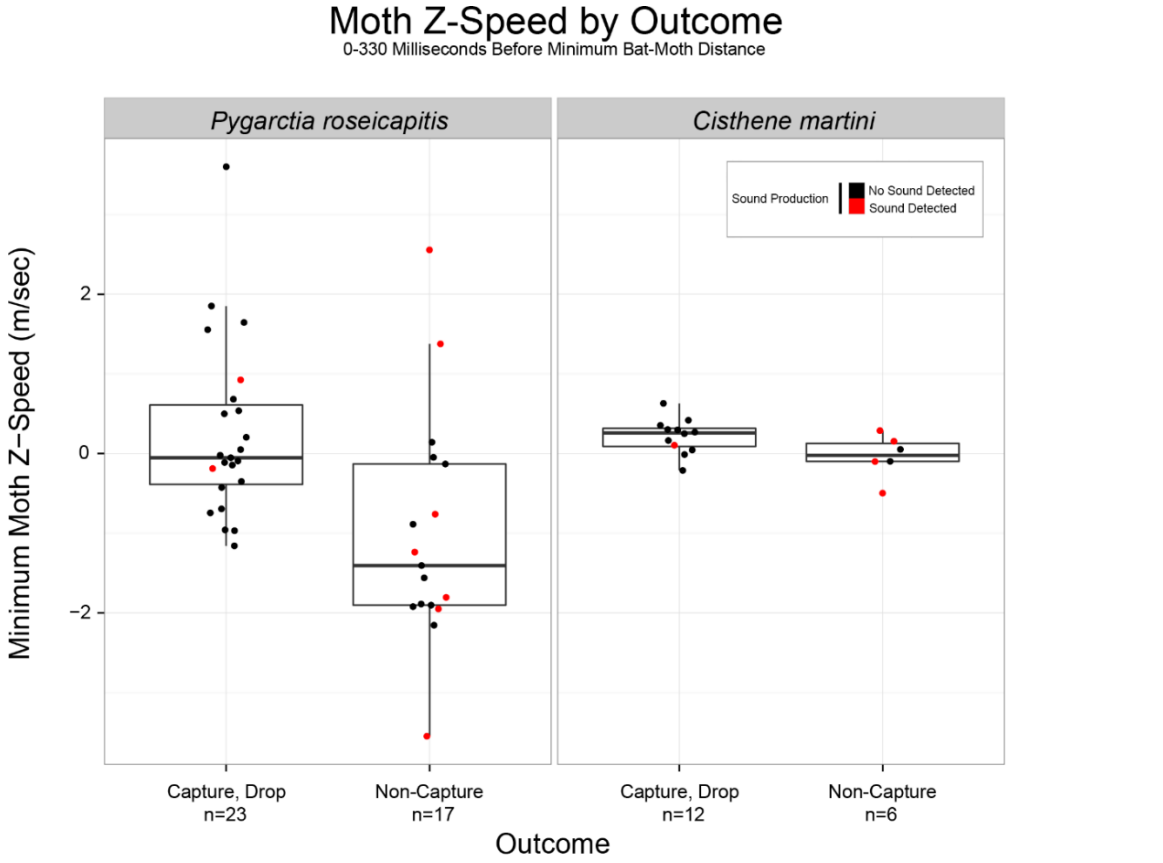

Minimum Bat-Moth Distance by Treatment and Outcome
Pygarctia roseicapitis
Cisthene martini
Minimum Bat-Moth Distance (cm)
100
10
1
TYMBALED, NON-CAPTURE
n=15
TYMBALED, CAPTURE
n=13
ABLATED, BOTH
n=12
TYMBALED, NON-CAPTURE
n=6
TYMBALED, CAPTURE
n=8
ABLATED, BOTH
n=4
Treatment and Outcome

Fig 6. Moth z-speed between "Capture, Drop" and "Non-Captured" outcomes among tymbaled (T+ and S groups) moths for *Pygarctia roseicapitis* and *Cisthene martini*. (Continued on Next Page).

Boxplot follows plotting conventions in Fig 3. The speed of the moths in the z-axis acts as a proxy for detecting diving evasive behavior. Positive values are upward flight, values near 0 $m*sec^{-1}$ are level flight, and negative values are downward flight. Only *P. roseicapitis* which were not captured were significantly different from 0 $m*sec^{-1}$, indicating that that species employed evasive dives. Neither outcome involving *C. martini* was significantly different from 0 $m*sec^{-1}$, indicating that this species did not frequently employ evasive dives. 3-D perspective plots display representative flight path data. Bats are depicted as larger points and moths as smaller points. Starting points are indicated by an arrow. Time flows from Yellow (Pre-Interaction) > Black (Interaction) > Purple (Post-Interaction). Black points are the closest distance between bat and moth and red points indicate when the moth was first detected to click. **(A)** "Non-Captured" *P. roseicapitis* diving (negative moth z-speed) in response to a bat attack. **(B)** "Non-Captured" *C. martini* taking no evasive action (moth z-speed ≈ 0) in response to a bat attack. Neither bat turned away from the clicking moth nor did they enact typical prey capture behaviors.


Moth Z-Speed by Outcome
0-330 Milliseconds Before Minimum Bat-Moth Distance
Pygarctia roseicapitis
Cisthene martini
Sound Production
No Sound Detected
Sound Detected
Minimum Moth Z−Speed (m/sec)
2
0
−2
Capture, Drop
n=23
Non-Capture
n=17
Capture, Drop
n=12
Non-Capture
n=6
Outcome

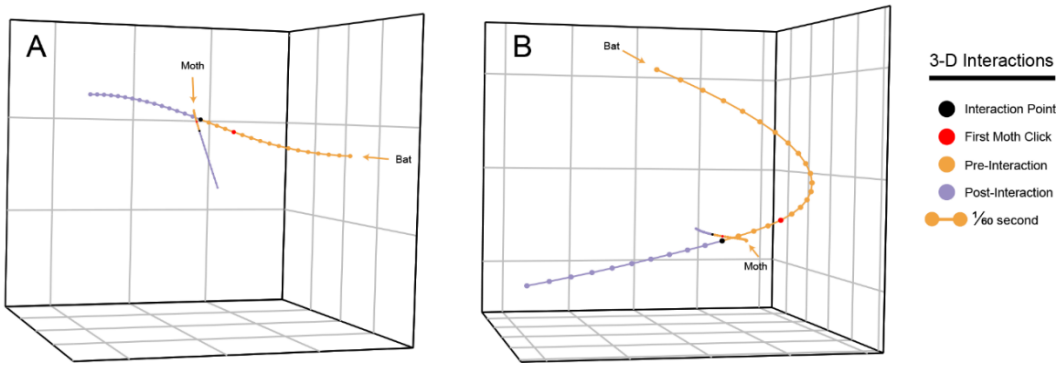

A
Moth
Bat
B
Bat
Moth
3-D Interactions
Interaction Point
First Moth Click
Pre-Interaction
Post-Interaction
1/60 second

Fig 7. Percentage of interactions hand-scored as "Evasion" by treatment for *P. roseicapitis* and *C. martini* (Continued on Next Page).

Numbers within each bar indicate the number of interactions observed for that treatment group and percentages indicate the percent of those observations that were scored as "Evasion".

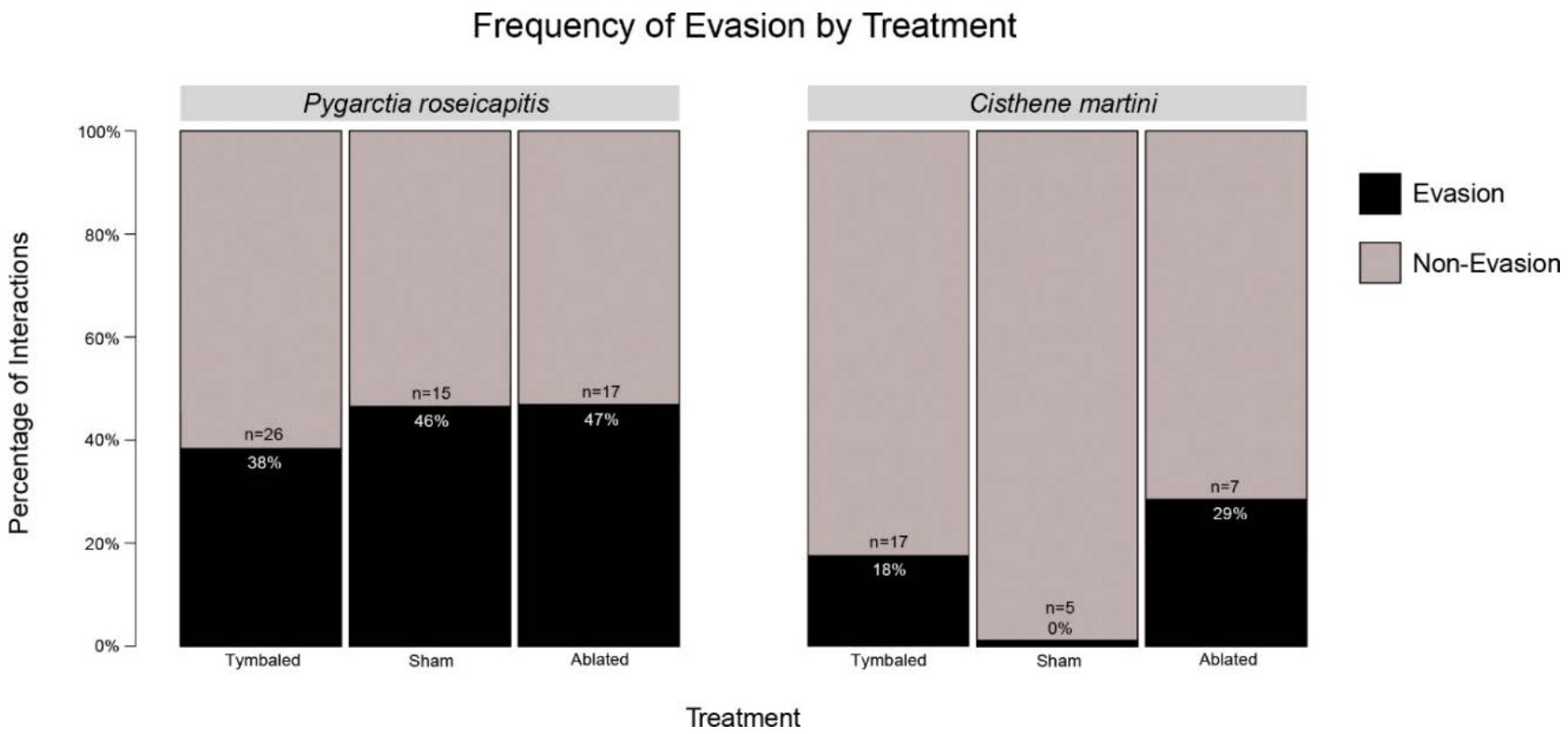

Frequency of Evasion by Treatment
Pygarctia roseicapitis
Cisthene martini
Percentage of Interactions
100%
80%
60%
40%
20%
0%
n=26
38%
n=15
46%
n=17
47%
n=17
18%
n=5
0%
n=7
29%
Tymbaled
Sham
Ablated
Tymbaled
Sham
Ablated
Treatment
Evasion
Non-Evasion

Fig 8. Ethogram showing the progression of bat attacks and possible outcomes of bat-moth interactions (Continued on Next Page).

A bat approaches a moth and can either capture or not capture that moth. If the moth is not captured that encounter has ended and the moth has survived. If the bat has captured a moth it can then either drop it or consume it. In this study's context, if a sound-producing moth is not captured it is evidence that aposematism was effective in deterring the bat attack. If a captured moth is rejected by being dropped they typically survive and this is evidence that defensive chemistry was effective in deterring consumption.

Bat Approaches
Capture
Non-Capture
(Aposematism Worked)
Consumed
(Nothing Worked)
Capture, Drop
(Chemical Defense Worked)

S1 Table. Breakdown of sample sizes used in each analysis (Continued on Next Page).

Data Subset	N	Figure	Breakdown By Species & Treatment					
			P. roseicapitis			*C. martini*		
			Tymbaled	Sham	Ablated	Tymbaled	Sham	Ablated
Filmed Interactions	167	2	50	12	25	33	30	17
↓								
Qualitative Evasive Response	87	7	26	15	17	17	5	7
↓			Tymbaled & Sham		Ablated	Tymbaled & Sham		Ablated
3-D Interaction Data	58	5 & 6	28		12	14		4
↓								
Moth Clicks Detected	20	Lines 128-135	14		0	6		0
↓								
Timing of Moth Clicks	15	3	9		N/A	6		N/A
↓								
Bat Calls Between Search Phases	19	4	6		6	5		2

CHAPTER II

NONCHALANT FLIGHT BEHAVIOR IN TIGER MOTHS (EREBIDAE: ARCTIINAE) IS CORRELATED WITH UNPALATABILITY

Research Article

Nonchalant flight behavior in tiger moths (Erebidae: Arctiinae) is correlated with unpalatability

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Abstract

Tiger moths (Erebidae: Arctiinae) are a subfamily of Lepidoptera which possess ultrasound-sensitive ears. These ears act as an early-warning system which can detect the ultrasonic cries of nearby echolocating bats, allowing the moths to enact evasive flight behaviors in an effort to escape predation. Our results demonstrate significant interspecific variation in the degree to which tiger moths utilize evasive flight behaviors to escape bat predators as well as in their degree of unpalatability. We provide evidence for the existence of a nonchalance continuum of anti-bat evasive flight response among tiger moths. We show that species are arrayed along this continuum based on their relative unpalatability to bat predators. Relatively unpalatable prey more often exhibit nonchalant flight behaviors whereas palatable prey more often employ evasive dives. Our findings demonstrate that the degree to which certain animals are protected by potent chemical defenses can influence the prevalence with which they exhibit evasive escape behaviors.

Keywords: Nonchalance, Evasive flight, Arctiinae, Anti-predator defense, Palatability

Introduction

Bats and insects have been locked in a coevolutionary arms race for nearly 65 million years (Conner and Corcoran, 2012). During this time, insects have converged on a number of adaptations that allow them to increase their odds of escaping predation. One major event was the development of ultrasound-sensitive ears that allow for the early detection of echolocating bats. Ears tuned to ultrasonic frequencies can be found in at least 5 orders of insects (Coleoptera, Lepidoptera, Mantodea, Neuroptera, and Orthoptera) and have evolved independently at least 14 times (Dawson, 2004; Greenfield, 2016). The ability to detect the ultrasonic cries of bats led to the development of avoidance behaviors such as negative phonotaxis, spiraling erratic flight, and power dives (Roeder, 1962). All five orders of ultrasound-sensitive insects contain reports of evasive flight in response to bat echolocation (Roeder, 1962; Miller and Oleson, 1979; Spangler, 1988; Yager at el., 1990; Dawson, 2004).

These maneuvers have been shown to be an effective means of dodging bat attacks (Acharya and Fenton, 1999; Triblehorn et al., 2008). However, there are potential costs to evasive maneuvers including energy expenditure, opportunity costs related to feeding or mating, or even exposure to terrestrial or aquatic predators (Guignion and Fullard, 2004; Yager, 2011).

Some tiger moths have been noted to lack any significant evasive flight response to bat attacks, even though they possess and utilize ultrasonic hearing (Acharya and Fenton, 1992; Dunning et al., 1992). Many tiger moths are able to produce ultrasound in response to bat echolocation to signal their unpalatability (Hristov and Conner, 2005; Barber et al., 2009; Dowdy and Conner, 2016). Their unpalatability is derived from sequestering

secondary plant substances from toxic host plants which they store in their own tissues (Boppre, 1984). Unpalatability varies between species and may relate to the concentration and type of chemical compounds they are capable of sequestering.

Field experiments with certain species of tiger moths have uncovered variation in the degree of nonchalant flight behaviors in response to bat attacks (Dowdy and Conner, 2016). Two sympatric tiger moth species, *Pygarctia roseicapitis* and *Cisthene martini*, differed in both the degree to which they enacted evasive dives as well as in their unpalatability. The more unpalatable *C. martini* was significantly more "nonchalant", performing dive maneuvers in only a few cases. In contrast, the less unpalatable *P. roseicapitis* was less nonchalant, diving much more frequently when attacked by bats. This suggested to us that a nonchalance continuum may exist. We hypothesized that more unpalatable tiger moth species would be more likely to exhibit nonchalant flight behaviors when attacked by bat predators.

Methods

Ethics Statement

No vertebrates (bats) were captured or handled during these experiments. All data involves free-flying bats in their natural habitats. No state or federal permits were required to conduct this work. The methods of this study were approved by the Wake Forest University Institutional Animal Care and Use Committee (protocol #A12-048). This work was performed with permission on private property.

Field Site

Field experiments were conducted at the Southwestern Research Station (SWRS) operated by the American Museum of Natural History. SWRS is located in Cochise County approximately 7 km southwest of Portal, Arizona, United States. The GPS coordinates of the field site are: 31°53'00.30" N 109°12'27.20" W; elevation: 1,650 m. This site was chosen for its high diversity of both bats and moths. The field trials were performed between July 18th and August 10th during 2011, 2012, and 2013.

Experimental Methods

The methods used to conduct this work follow those previously published. Detailed information about moth collection, bats species identification, how field trials were conducted, audio recording and videography, and palatability can be found in a previous publication (Dowdy and Conner, 2016). To reduce the effects of pseudo-replication, we used only the first interaction for each moth included in our study.

Evasive Flight Behavior

We recorded interactions of moths with free-flying bats to determine how frequently they utilized evasive flight maneuvers. We manually classified each interaction and scored them as either "Turn Away", "Dives", or "No Evasion" as defined in classic studies of moth evasive flight (Roeder, 1962). As in previous studies, we did not observe frequent "Turn Away" behavior (Dowdy and Conner, 2016). For this reason, we restricted our analysis to include only moths exhibiting "Dives" (renamed "Evasion") or "No Evasion". We included data about evasive flight behaviors from both clicking and silent moths in this study.

Palatability

All moths studied here were found to produce ultrasound in response to bat echolocation. Therefore, all palatability reported in this study was measured from silent moths that had their sound-producing structures removed via ablation as described in previous studies (Corcoran and Conner, 2012; Dowdy and Conner, 2016). This was done to avoid confounding true palatability with the possible deterrent effects of moth clicks. In addition, all palatability comes from interactions with free-flying bats. When captured by bats, moths were either dropped immediately ("Capture, Drop" - unpalatable) or not at all ("Consume" - palatable).

Bertholdia trigona Data

Data for *B. trigona* come from a previous study using similar methods as those used in this study (Corcoran and Conner, 2012). Palatability and evasive diving flight data for *B. trigona* come from Fig. 2B and Fig. 4A, respectively (Corcoran and Conner, 2012). We included only data about diving evasive behavior from this study. Palatability data was taken only for silenced moths and was measured as the number of captured individuals that were and were not consumed.

Pygarctia roseicapitis and Cisthene martini Data

Data for *P. roseicapitis and C. martini* come from a previous publication (Dowdy and Conner, 2016). Palatability and evasive diving flight data for these species come from Fig. 2 and Fig. 7, respectively. Some additional, new palatability data for these species is included in this report.

Statistics

Statistical analyses were performed in R version 3.3.2 (R Core Team, 2016). We used generalized linear models with a binomial variance function and logit link function to compare the proportion of nonchalant flight behavior between species. This approach was also used to compare the proportion of unpalatability between species. We used these proportions as a property of each species and examined how unpalatability predicted nonchalant flight using linear regression. We constructed 95% prediction intervals for nonchalant flight using the predict function in R.

Results

We found significant variation in nonchalance and unpalatability between species (**Fig. 1**). Significance, level contrasts, and coefficients are given in **Table 1 and 2**. *Pygarctia murina* was significantly less nonchalant than all other species in our analysis. The prevalence of nonchalance between *B. trigona* and *P. roseicapitis* were not significantly different, though *B. trigona* was significantly less nonchalant as compared to *Carales arizonensis* and *C. martini*. *P. roseicapitis* and *C. arizonensis* were not significantly different, however *P. roseicapitis* was significantly less nonchalant than *C. martini*. Finally, *C. arizonenesis* and *C. martini* were not significantly different.

P. murina and *B. trigona* were significantly less unpalatable than all other species in our analysis. *C. arizonensis* and *C. martini* were significantly more unpalatable than all other species in our analysis. *P. roseicapitis* was significantly different from both of these groups, exhibiting an intermediate level of unpalatability.

Modeling nonchalance from unpalatability yielded a significantly strong, positive relationship ($p<0.05$, Adj. R^2: 0.87; **Table 3**). However, because we could only sample 5

species, our 95% prediction intervals are very large, making our estimate of nonchalance from unpalatability imprecise (**Fig. 2**).

Discussion

These results show that, at least in tiger moths, significant interspecific variation in the likelihood of enacting evasive maneuvers exists. We define this as a nonchalance continuum, with species exhibiting dives infrequently being classified as more nonchalant. Additionally, there are significant interspecific differences in palatability among tiger moths. Variation in palatability has been noted in other insects, and is generally known as a "palatability spectrum" (Brower et al., 1972; Turner, 1984).

Our results demonstrate that where species lie along the nonchalance continuum and the palatability spectrum are significantly related. Less unpalatable moths face an increased risk of consumption if captured by bat predators, and so they utilize evasive diving maneuvers more frequently in an effort to offset some of this risk. Interestingly, previous studies have reported that palatability also relates to evasive behaviors enacted from rest on substrates (*e.g.*, flying away, dropping from vegetation) in response to simulated bird predation (Evans, 1983). These experiments describe unpalatable prey exhibiting behaviors that could also be considered more nonchalant.

Of course, tiger moths are not the only chemically protected insects that employ evasive flight to contend with bat predation. Each of the 4 orders of insects known to hear and evade bat predators contain examples of species utilizing a chemical defense of some kind (Eisner et al., 2005; see **Introduction**). Intraspecific variation in chemical protection

likely exists in these insect lineages as well, and therefore it should not be unexpected to find variation in nonchalance beyond the tiger moths.

The relatively palatable *P. murina* produces anti-bat ultrasound with acoustic characteristics similar to those of its more unpalatable, sympatric congener *P. roseicapitis* (Dowdy and Conner, 2016; Dowdy, unpublished). This is similar to the relationship between *Cycnia tenera* and *Euchaetes egle*, two sympatric tiger moths native to eastern North America. Lab experiments with naïve bats have shown that the palatable *E. egle* was protected from predation by *Eptesicus fuscus* bats, acting as an acoustic Batesian mimic of *C. tenera* (Barber and Conner, 2007). It is possible that *P. murina* is an acoustic Batesian mimic of *P. roseicapitis* as well as other toxic, acoustically aposematic tiger moth species. It is intriguing then, that *P. murina* is less nonchalant than its more toxic model. It is possible that Batesian mimics will often "play it safe" by diving more frequently than their honestly signaling counterparts. This should be explored further with experiments confirming model-mimic relationships followed by a comparison of the frequency of evasive behaviors between these two groups.

By gathering more evasive flight and palatability data for more species, we could also better predict nonchalance from palatability data. This is important, as measuring palatability is often easier than making detailed observations of evasive flight. Palatability may even be measurable using methods like mass spectroscopy to detect the defensive toxins and their concentration directly (Bowers, 2009; Anderson et al., 2016). Gaining a better understanding of the variation in nonchalance between insects could help us understand how animals balance the potential costs and risks of utilizing evasive flight maneuvers with the potential benefits gained from escaping bat predators.

Conclusion

Many anecdotal reports of sluggishness or 'fearlessness' among chemically defended, aposematic animals exist. Wallace noted of the skunk that, "Its consciousness that it needs only to be seen to be avoided gives it that slowness of motion and fearlessness of aspect which are, as we shall see, characteristic of most creatures so protected" (Wallace, 1889). Our results corroborate these reports, demonstrating that the degree to which certain animals are protected by potent chemical defenses can influence the prevalence with which they exhibit evasive escape behaviors.

Acknowledgments

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Figure 1. Interspecific Variation in Nonchalant Flight Behavior and Unpalatability (Continued on Next Page). Sample sizes for each category and percentage exhibiting nonchalant flight or unpalatability are given in each bar. Significantly different groups within each plot are indicated by different letters (see Tables 1 and 2 for more info).

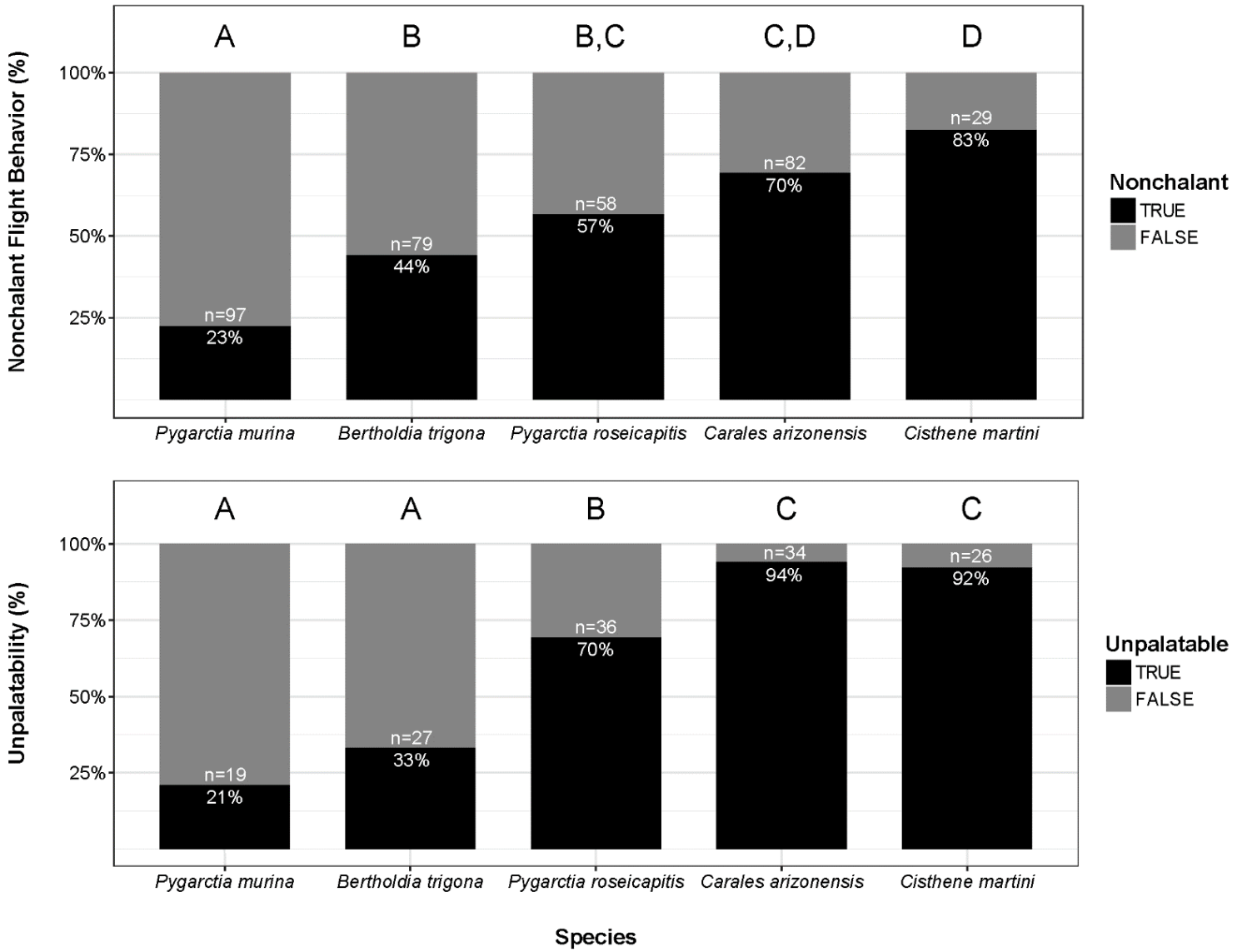

A
B
B,C
C,D
D
100%
75%
50%
25%
Nonchalant Flight Behavior (%)
n=97
23%
n=79
44%
n=58
57%
n=82
70%
n=29
83%
Pygarctia murina
Bertholdia trigona
Pygarctia roseicapitis
Carales arizonensis
Cisthene martini
Nonchalant
TRUE
FALSE
A
A
B
C
C
100%
75%
50%
25%
Unpalatability (%)
n=19
21%
n=27
33%
n=36
70%
n=34
94%
n=26
92%
Pygarctia murina
Bertholdia trigona
Pygarctia roseicapitis
Carales arizonensis
Cisthene martini
Unpalatable
TRUE
FALSE
Species

Table 1. Generalized Linear Model Results for Nonchalant Flight. GLM model used nonchalant flight presence/absence as response variable and species identity as predictor variable. Values in parentheses are standard errors on coefficient estimates.

EVASIVE FLIGHT MODEL	Contrast 1	Contrast 2	Contrast 3	Contrast 4	Contrast 5
(Intercept)	-1.23^{***}	-0.23	0.28	0.82^{***}	1.57^{**}
	(0.24)	(0.23)	(0.27)	(0.24)	(0.49)
Pygarctia murina		-1.00^{**}	-1.50^{***}	-2.05^{***}	-2.80^{***}
		(0.33)	(0.36)	(0.34)	(0.55)
Bertholdia trigona	1.00^{**}		-0.51	-1.05^{**}	-1.80^{***}
	(0.33)		(0.35)	(0.33)	(0.54)
Pygarctia roseicapitis	1.50^{***}	0.51		-0.55	-1.29^{*}
	(0.36)	(0.35)		(0.36)	(0.56)
Carales arizonensis	2.05^{***}	1.05^{**}	0.55		-0.74
	(0.34)	(0.33)	(0.36)		(0.55)
Cisthene martini	2.80^{***}	1.80^{***}	1.29^{*}	0.74	
	(0.55)	(0.54)	(0.56)	(0.55)	
AIC	429.17	429.17	429.17	429.17	429.17
BIC	448.38	448.38	448.38	448.38	448.38
Log Likelihood	-209.58	-209.58	-209.58	-209.58	-209.58
Deviance	419.17	419.17	419.17	419.17	419.17
Num. obs.	345	345	345	345	345

$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

Table 2. Generalized Linear Model Results for Unpalatability. GLM model used a binary measure of unpalatability as the response variable and species identity as predictor variable. Values in parentheses are standard errors on coefficient estimates.

PALATABILITY MODEL	Contrast 1	Contrast 2	Contrast 3	Contrast 4	Contrast 5
(Intercept)	−1.32*	−0.69	0.82*	2.77***	2.48***
	(0.56)	(0.41)	(0.36)	(0.73)	(0.74)
Pygarctia murina		−0.63	−2.14**	−4.09***	−3.81***
		(0.70)	(0.67)	(0.92)	(0.93)
Bertholdia trigona	0.63		−1.51**	−3.47***	−3.18***
	(0.70)		(0.55)	(0.84)	(0.84)
Pygarctia roseicapitis	2.14**	1.51**		−1.95*	−1.66*
	(0.67)	(0.55)		(0.81)	(0.82)
Carales arizonensis	4.09***	3.47***	1.95*		0.29
	(0.92)	(0.84)	(0.81)		(1.04)
Cisthene martini	3.81***	3.18***	1.66*	−0.29	
	(0.93)	(0.84)	(0.82)	(1.04)	
AIC	137.56	137.56	137.56	137.56	137.56
BIC	152.34	152.34	152.34	152.34	152.34
Log Likelihood	-63.78	-63.78	-63.78	-63.78	-63.78
Deviance	127.56	127.56	127.56	127.56	127.56
Num. obs.	142	142	142	142	142

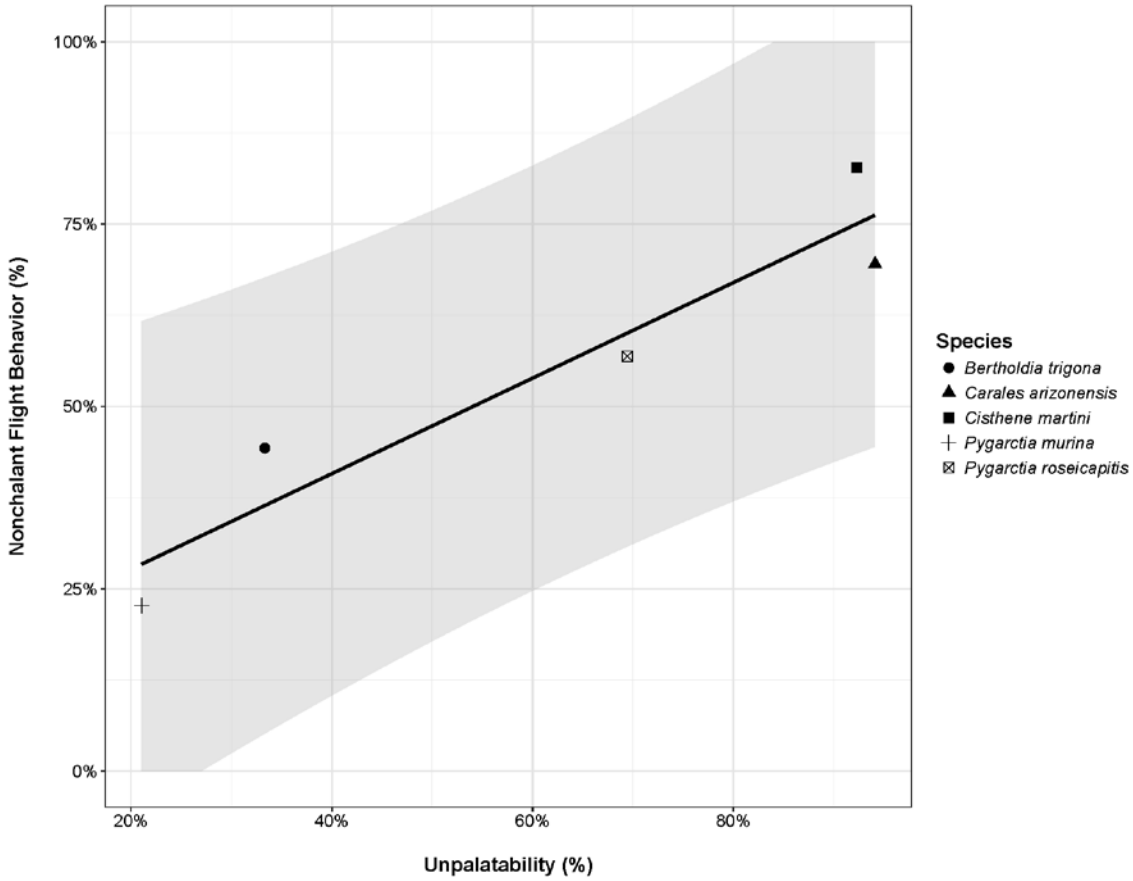
$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

Table 3. Linear Model of Nonchalance Results. LM model used the nonchalance estimate for each species the predictor and the unpalatability estimate for each species as the response variable. Nonchalance and unpalatability estimate values are proportions of individuals within each species exhibiting each trait. Values in parentheses are standard errors on coefficient estimates.

	Nonchalance
(Intercept)	0.15
	(0.09)
Unpalatability	0.66*
	(0.12)
R^2	0.90
Adj. R^2	0.87
Num. obs.	5
RMSE	0.08

$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

Figure 2. Linear Model of Nonchalance. Grey ribbon represents the 95% prediction interval for the linear model (Table 3). For a given Unpalatability, Nonchalance is expected to fall within these intervals in 95% of cases.



CHAPTER III

CAN CHARACTERISTICS OF TIGER MOTH (EREBIDAE: ARCTIINAE) ANTI-BAT SOUNDS BE PREDICTED FROM MORPHOLOGY?

Research Article

Can characteristics of tiger moth (Erebidae: Arctiinae) anti-bat sounds be predicted from morphology?

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Abstract

Tiger moths use modified cuticular plates called “tymbal organs” to produce ultrasonic sounds when attacked by echolocating bats. These sounds protect the moths from predation by signaling toxicity or disrupting bat echolocation. The morphology of the tymbal organs and the sounds they produce vary greatly between species, but it is unclear how the variation in morphology gives rise to the variation in acoustic characteristics. We first review the literature and summarize what is known about both tymbal morphology and sound production in tiger moths. This is the first study to measure the morphology of tymbals and the ultrasonic signals they produce simultaneously. We use linear modelling to predict how the morphological features of tymbals give rise to their acoustic properties. We show that the number of striations on the tymbal’s surface (“microtymbals”) and to a lesser extent the ratio of tymbal to thorax surface area have a strong positive correlation with the number of clicks a moth produces per unit time. We also found that separate monophyletic clades have significantly different regression coefficients, thus the relationship between microtymbals and click rate is dependent on the phylogenetic position of different species. This predictive model will allow us to estimate the click rate of moths from preserved material, such as that in natural history collections, in cases where traditional recording methods are too costly or difficult to undertake. This will greatly accelerate our understanding of the distribution of sound production and possibly the acoustic anti-bat strategies employed by tiger moths worldwide.

Keywords: Arctiinae, tymbal, sound production, morphology, anti-bat defense

Introduction

Tiger moths (Erebidae: Arctiinae) are a charismatic and diverse lineage of moths, well-known for their ability to detect sound and produce sound in response to bat echolocation, tactile stimulation, and to the pheromones and/or sounds of conspecifics during sexual courtship (Conner, 1999; Conner and Corcoran, 2012). The acoustic detection of foraging bat predators and potential mates is made possible through the use of ultrasound-sensitive ears located on the thorax (Roeder and Treat, 1957; Yack and Dawson, 2008). In response to these acoustic stimuli, the moths produce sound to either signal their toxicity or unprofitability to bat predators (acoustic aposematism), mimic the acoustic aposematic displays of other species (acoustic mimicry), disrupt the echolocation of attacking bats, rendering the moth difficult to capture (sonar jamming), as a necessary component of sexual courtship, or some combination of these functions (Sanderford and Conner, 1990; Barber and Conner, 2007; Barber et al., 2009; Corcoran et al., 2009; Corcoran and Conner, 2012; Dowdy and Conner, 2016).

These sounds are produced using two "tymbal organs" which are modified cuticular plates enclosing an air-filled cavity. The tymbals are located on both sides of the thorax on the third metepisternite and can be completely or partially covered in scales (De Villiers, 1832; Hinton, 1955). They are composed of a transparent or semi-transparent blistering of the exoskeleton whose surface may be smooth or include an anterior or medial band of parallel corrugations or folds. This band is known as the "striated band" and the corrugations are called "microtymbals" (Forbes and Franclemont, 1957). The microtymbals can be classified as either major grooves, which each contain

the socket of a single scale, or minor grooves which can occur between major grooves and are often spaced out more irregularly, resembling wrinkles.

Tymbal morphology was once considered a potentially informative character for use in systematics, but it was later discovered that the character is absent in many genera, pointing to the possibility that tymbal development for sound production is lost frequently or is a highly plastic character (Forbes and Franclemont, 1957). However, this variability seems to depend on the genus in question, as other studies have noted little variation within certain genera (Adams, 1991; Rawlins, 1982; Watson, 1975).

The earliest published account of sonic emissions from a tiger moth comes from the European species *Cymbalophora pudica*, which produces human-audible sounds during flight (De Villiers, 1832). Later reports showed that sound was also produced by other tiger moths like *Miltochrista miniata* when physically restrained and by *Setina aurita* which was found to produce sound in sexual courtship in a manner similar to *C. pudica* (Haldeman, 1848; Guenee, 1861; Guenee, 1864; Laboulbene, 1864). Originally, it was thought that the tymbal functioned either by compression of the thorax during flight or as a stridulatory organ which could be made to produce sound by rubbing the leg against it (De Villiers, 1832; Laboulbene, 1864; Carpenter, 1938; Forbes and Franclemont, 1957; Hartland Rowe, 1959). However, moths which had their legs removed were found to retain the ability to produce sound (Peter, 1911). Detailed observation and dissection of the tymbal and surrounding tissues concluded that the metathoracic basalar musculature directly attaches to the tymbal and is responsible for buckling the surface of the tymbal, causing sounds to be produced (Blest et al., 1963). Later examinations determined that at least three muscles, the tymbal muscle (pv2) and

two accessory muscles (pv1 and/or pv6), are implicated in the functioning of the tymbal (Fullard and Heller, 1990).

The contraction of these muscles causes the tymbal to buckle along the length of the striated band beginning from the dorsal end. As the buckling proceeds through each microtymbal, discrete clicks are produced. In some cases, tymbals lacking microtymbals can still be functional, with the entire surface of the tymbal producing a single click from the buckling action. After contraction, the muscle relaxes and the inherent elasticity of the tymbal organ reverses the buckling, again producing a single click for each microtymbal. These "modulation cycles" of contraction and relaxation repeat a number of times, producing trains of clicks (Blest et al., 1963).

The activation of the tymbal organ appears to be controlled by a metathoracic reflex arc connecting the tymbal musculature with tympanal auditory receptors, and even functions in response to tactile and acoustic stimulation after decapitation (Blest et al., 1963; Fullard, 1982). However, because the tymbals can also be activated in response to tactile stimulation and in some cases chemical stimuli, it is likely that other interneurons, including a tymbal central pattern generator, also control their action (Fullard and Heller, 1990).

Depending on the species, the sounds produced by tiger moths can also vary greatly in frequency, intensity, the timing of presentation relative to bat attack echolocation, the number of clicks produced per modulation cycle, and many other characteristics (Barber and Conner, 2006; Corcoran et al., 2010). Sound emitter morphology is known to have a strong influence over the acoustic qualities of sonic emissions in a variety of animal groups (Aves: Palacios and Tubaro, 2000; Pisces: Kéver

et al., 2012; Anurans: McClelland et al., 1996; Mammals: Charlton and Reby, 2016). Though the general mechanism of sound production in tiger moths is well understood, the tymbal properties that give rise to the variation in their sonic characteristics are still a mystery. Previous studies have focused on either examining variation in tymbal morphology or in the sounds they produce, but how the two are related has never been quantitatively studied. We believe it is imperative to determine whether and how precisely sound production can be predicted from morphological analysis of the tymbal organs of tiger moths, as this would be a much more cost-effective method of surveying sound production. This method could have the additional benefit of allowing aspects of sound production to be inferred for species that have recently gone extinct or perhaps even from fossilized material with intact tymbal organs (Douglas and Stockey, 1996).

Currently, the best method we have for determining the acoustic properties of anti-bat tiger moth sounds is to record them directly from living animals. This requires at a minimum 1) expensive audio and computing equipment, capable of recording and reproducing sounds containing ultrasonic frequencies up to 250kHz, 2) high-quality recordings of appropriate bat echolocation attack sequences to stimulate moth sound production, 3) a high level of expertise in both operating acoustic equipment and analyzing acoustic data, 4) knowledge of where target moth species can reliably be found, 5) permits to collect and conduct research with living animals, 6) travel to remote locations where living specimens can be found, and finally no small amount of luck in finding and eliciting a response from individuals of the target species. Alternatively, it may be possible to infer the acoustic properties from tymbal morphology, requiring at a minimum: 1) preserved specimens with intact thoraces and tymbal organs, 2) the ability

to examine and measure aspects of tymbal morphology, and 3) knowledge of how those measures relate to certain aspects of sound production. This study addresses the third requirement.

We begin by surveying the literature beginning with the discovery of tymbalar sound-production in tiger moths nearly two centuries ago in an attempt to consolidate and quantify our knowledge about tiger moth sound production and tymbal morphology.

We examined four major morphological features related to the tymbal organ including the tymbal surface area, the thorax surface area, the ratio of tymbal and thorax surface area, and the number of microtymbals. Because each microtymbal is thought to contribute a single click to the active and passive half-modulation cycles we hypothesized that the overall number of microtymbals would have a strong effect on the number of clicks a moth produces per second ("click rate"). Another influence on click rate is how quickly modulation cycles are completed and the amount of time it takes to begin the next cycle. We expect that these qualities will be dependent on the tymbal musculature and aspects of the nervous system controlling those muscles. These are difficult to measure directly, so we use measures of tymbal and thorax size as a proxy. We hypothesize that that these proxy measures of tymbal musculature will be positively correlated with click rate.

We present here the first study to examine tymbal morphology and tymbal sounds simultaneously for a large number tiger moth species in an effort to determine how the morphology of the tymbal organ determines the click rate of tiger moth sounds. These results represent a crucial first step toward inexpensively predicting the acoustic

characteristics and possibly the defensive functions of tiger moth anti-bat sounds on an unprecedented scale utilizing preserved museum specimens.

Methods

Literature Review

We searched the literature for published accounts of tiger moth sound production and tymbal morphology. For each of these articles and the articles cited in their bibliographies, we gathered data about tymbal morphology and sound production. From each study we recorded: (A) the citation name, (B) the published binomial name, (C) a summary of the data relating to tymbal morphology if present (*e.g.*, "35 microtymbals present"), (D) quality of tymbal morphology information coded into 5 levels, (E) a summary of the data relating to sound production if present (*e.g.*, "Sound not produced in response to tactile stimulation"), (F) information about stimulation type used coded into 4 categories, and (G) information about the recording technique used coded into 4 categories.

The quality of tymbal morphology data (D) was coded as: (0) no information, (1) binary measure (*i.e.*, microtymbals present / absent), (2) microtymbals described in qualitative categories (*e.g.*, "well-developed" or "shallow or weak", (3) actual counts given (*e.g.*, "35 microtymbals"), or (4) tymbal with microtymbals imaged (*e.g.*, SEM, drawing, or photograph included). The stimulation type employed in each study (F) was coded as: (0) no stimulation, (1) tactile stimulation, (2) simulated or real bat echolocation, (3) courtship scenario. The recording technique used in each study (G) was coded as: (0) no recording made, (1) other method employed (*e.g.*, human hearing, visual examination

of tymbal organ movement), (2) sound was monitored using acoustic equipment, but not recorded, (3) sound was recorded using digital or analog acoustic equipment.

Often, well-studied species would have data presented in multiple studies. In these cases, we included a description of the data from whichever article contained information from the highest quality category. For instance, if one article presented presence or absence information about microtymbals and a second article presented a count of microtymbals and an SEM of the organ, we retained all citation information, but included in our summary only the count of microtymbals and a reference to the figure containing the SEM from the second article. If two studies contained conflicting information we included both descriptions regardless of quality and indicated the source from which each description came.

We included only records that were ascribed to an explicitly stated binomial name, though we retained cases where "sp." was used for the species identifier. If different studies referred to a "sp." within the same genus, we counted these as unique species because this only occurred between studies conducted in different locations and thus we believe these were unlikely to be the same species. We also retained accounts for subspecies as unique entries.

We removed from our analysis any species identifications that could not be verified as valid names through online services such as The Global Lepidoptera Names Index (Natural History Museum, London, UK) though we kept them in our table under the name "NO MATCH" and included a reference to their published names. Where necessary, the published names for each record were updated to their currently accepted

taxonomic identifications based on information from these online resources prior to further analyses.

In one case a reference (Rawlins, 1982) contained data for two species (*Bertholdia braziliensis, B. soro*r) that we found to have been later synonymized, but the data given were quite different. We chose to keep these as separate entries denoting them as "*B. soror* 1" and "*B. soror* 2". We were aware of a single report which falsely identified a Geometrid moth (*Eubaphe unicolor*) as a tiger moth (*Virbia fragilis*) (Corcoran, pers. comm.). We kept this record in our table so that the clarification of its identity could be published, but removed it from our analyses. Finally, some accounts only summarized results for a genus overall, rather than providing individual species records. We opted to include the data under the given genus name with the species identified as "spp." to indicate that it includes information for multiple species. In these cases, we included the published accounts in our analyses at the generic level, but did not count these as unique species records in our analyses.

For our analysis of the presence or absence of sound among genera and species, we coded sound as present whenever a moth was noted as producing sound in any context. Whenever two studies disagreed we coded this as a third category, "Disagreement". Based on our own research, we believe the most likely reason for disagreement between studies can be explained by false negative responses, which can be common when proper recording conditions are not used. We hesitate to dismiss these results completely however, as it is possible that they reflect real differences between individuals of the same species in different geographic locations, differences in response

between the sexes, the presence of cryptic species that are morphologically similar, but which differ in their sound production, or some other phenomenon.

Field Site and Insect Capture Method

Field experiments were conducted at the Yanayacu Biological Station and Center for Creative Studies (YBS) approximately 5 km west of Cosanga, Ecuador. The GPS coordinates of the field site are: 00°36.235' S, 77°52.917' W; elevation: 2,100 m. This location was chosen based on the impressive amount of information available regarding the many moth species present there (Rab-Green et al., 2011). YBS lies on the eastern slopes of the Andes and is comprised of primary forest as well as partially reforested pastures and roadsides.

Insects were collected on station grounds from sheets illuminated with 15 Watt ultraviolet “quantum” lights (Leptraps.com; F15T8QBL). Moths were collected August 21th-29th, 2013 and placed individually in 30mL plastic containers and stored for up to 24 hours at ambient outdoor temperatures (12-15° C) prior to acoustic recordings. We did not collect enough individuals of each sex within our collected species to examine sexual differences in click rate and microtymbal numbers. Because all of our collected moths were collected using the same method, at the same location, during the night, and while bats were actively hunting nearby, we did not treat males and females differently in these analyses.

A subset of our data included moths from both Arizona and North Carolina field sites. This was done to extend our analysis to a broader geographic range and to include measurements of some of the classic, well-studied species from previous research efforts.

Arizonan moths included were captured at the Southwestern Research Station (SWRS) operated by the American Museum of Natural History. The GPS coordinates of the field site are: 31°53’00.30” N, 109°12’27.20” W; elevation: 1,650 m. North Carolinian moths included were captured on private property with permission of the property owner at a location approximately 4.5 km north west of Elk Knob State Park (36°19'57.44"N, 81°41'44.38"W).

Acoustic Recordings

Freshly captured moths were held by the wings, which were folded above the thorax and restrained with a hemostat. All recordings were made in a darkened room at night in ambient outdoor temperatures (~12° C). An Avisoft Bioacoustics USGH digital recording unit was connected to a single Avisoft CM16/CMPA ultrasonic microphone (± 3 dB from 15-140 kHz) and set to record at a sampling rate of 250-500 kHz. The microphone was placed perpendicular to the midline of the moth body, 10 cm from the thorax of the individual (where the sound-producing organs are located). We recorded mainly from the ipsilateral tymbal (with respect to the microphone), though acoustic emissions from the contralateral side were detected in our recordings at lower intensity relative to those of the ipsilateral side. An AT100 ultrasonic speaker (Binary Acoustic Technology) was placed 10 cm from the posterior end of the moth thorax (where the tympanal hearing organs are located), parallel to the midline of the body. Moths were stimulated to produce sound by playing a pre-recorded echolocation attack sequence from the insectivorous bat, *E. fuscus*. This species of bat was chosen because it is one of the few bat species sympatric with all moth species included in this study (Arguero and Albuja, 2012; Miller et al., 2016). Search, approach, and buzz phases of bat echolocation

were all present and spanned a pulse interval of 115 ms in search phase to 6 ms in the buzz phase. Echolocation intensity reached and then sustained a peak equivalent Sound Pressure Level of 100 dB at 10 cm in the approach phase. For more details see previously reported methods (Barber and Conner, 2006). Stimuli were repeated seven times per individual with approximately 4–5 seconds of silence between trials. Files were saved in a .WAV format. Each recording contained only a single simulated bat attack.

Specimen Vouchering

After acoustic assays were completed each specimen was euthanized in a freezer (~-20° C) for 24 hours. Afterwards, the specimens were thawed and field pinned. Each specimen was pinned on top of an 18% grey card and the wings were spread and pinned in place with insect pins. A metric photographic scale (1mm increments) was placed next to each specimen. We photographed the dorsal and ventral sides of each specimen using a Canon XTi DSLR (10.1 MP; RAW image format; shutter speed: 1/250 sec) with Canon EF-S 60mm Macro Lens (manual aperture of f/11) and a Canon MT-24EX Macro Twin Lite Flash for illumination. Once photographs were taken we removed the legs, antennae, proboscis, abdomen, and wings and placed each into separate 1.5mL tubes filled with 95% EtOH or glassine envelopes. The thorax and head were then placed into their own 1.5mL tube filled with 95% EtOH. All tissues were stored at -80° C and are currently archived at Wake Forest University.

Scanning Electron Microscopy

We used a scanning electron microscope (SEM) (Model: Amray 1810) to image the tymbal organs. To prepare the specimens for imaging we removed each thorax from

its 1.5mL tube and evaporated the EtOH by air drying for 15-30 minutes under a fan. We found that critical point drying was not necessary for these specimens. To make the tymbal and microtymbals more clearly visible and easily countable we used a combination of compressed air, scotch tape, and forceps to remove the scales from the surface of the tymbal and thorax taking care not to damage or puncture the tymbal surface. We also removed the mesepisternum and/or mesepimeron to make imaging the anterior edge of the tymbal easier. The specimens were placed on stubs with double-sided carbon tape and were gold coated in a sputter coater (Model: Cressington Scientific Sputter Coater 108) for 30 seconds under argon gas. Images were taken using an acceleration potential of 10-12 kV and saved as .TIF. Only a single side of each specimen was imaged. One image was taken as a direct side-on view of the body such that both the thorax and tymbal organ could be seen. A second image zoomed in on the tymbal was taken to facilitate the counting of the microtymbals (**Fig. 1 A, B**).

Image Analysis

Images from SEM were analyzed in Adobe Photoshop CC. First, two separate layers were created for the tymbal and the thorax. The tymbal and thorax were outlined in their respective layers using the Paintbrush Tool and filled in (**Fig. 1 B, D**). Our thorax measurements do not include the coxa of the first thoracic segment nor the patagium, but they do include the coxa of the second and third thoracic segments and the entirety of the scutellum. The Ruler Tool was used to set the scale between pixel and millimeters using the scale bar embedded in each image from the SEM image capture software. Each layer was selected using the Magic Wand Tool and the Record Measurements button yielded the surface area measures for the tymbal and thorax. The second zoomed image was used

to count the number of visible microtymbals. We define microtymbals as deep or shallow depressions in the surface of the tymbal along the striated band which also have a corresponding hair-socket which we dub “major grooves”. Some specimens exhibited wrinkles or “minor grooves” that occurred between major grooves. We disregarded these in this analysis. We felt it necessary to distinguish between these two types of “microtymbals” as it is not clear whether the minor grooves contribute to sound production. When microtymbals were not present we counted that as 0 microtymbals.

Acoustic Analysis

Click Detection and Measurement

We used Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) to detect and measure the number of moth clicks present in each of our recordings. For each .WAV file we generated a spectrogram with the following frequency resolution attributes: FFT length = 256, Frame Size = 50%, Window = FlatTop with a window overlap of 96.87% (8 samples). We then used the Automatic Parameter Measurements tool to automatically identify the moth clicks in our files. To do this, we used a two-threshold approach. The threshold defining when a signal should be classified was variable depending on the intensity of the individual moth. Our second threshold defining the end of a detected signal was -8dB relative to the peak intensity of that signal. After processing each file with the automatic method, we manually went through and removed spurious results, manually included clicks that were not detected, and manually separated individual clicks when multiple clicks occurred too close together in time and were classified as the same signal. The timestamps of each click were saved into a .CSV file for further analysis.

Measuring Maximum Click Rate

This study uses the maximum click rate produced by a given moth as a measure of the rate of its sound production. This was chosen because it is less sensitive to the possible incomplete activation of the tymbal organ. Maximum click rate is defined as the largest number of clicks present in a 100 ms time window, multiplied by 10 for reporting in terms of the number of clicks produced per 1 second. Hereafter we refer to maximum click rate as simply "click rate" or "CR". To measure CR, we wrote a custom R script which took as its input the .CSV files generated in SASLab Pro. This script starts from the first detected click in a recording and counts the number of detected clicks that occur within 100 ms. In further iterations, this 100 ms time window is shifted by a single click event and the click rate is recalculated. Once the window reaches the final click in a recording the maximum recorded click rate is determined and reported for a given recording. Maximum click rate measurements from multiple simulated bat attacks against the same moth are then compared and the overall maximum is retained and reported as "CR".

Linear Regression Model Selection

Model Selection

We measured three aspects of tymbal morphology: (1) the number of microtymbals ("MT"), (2) the tymbal surface area ("TYSA") expressed in mm^2, and (3) the thorax surface area ("THSA") expressed in mm^2. We also calculated and included (4) the ratio of tymbal surface area to thorax surface area ("T2T"). We modeled the effect that these four chosen predictors had on the maximum click rate ("CR"). We included

MT to test whether they have a direct effect on increasing CR. We included TYSA, THSA, and T2T as a proxy for the size of the musculature that drives the tymbal organ. We hypothesized that larger tymbals, larger thoraces, and tymbals that were relatively large for their thorax size would be more likely to have larger tymbal musculature, which would generate a higher CR. We examined a correlation matrix between all measured variables to determine which should be included in our model (**Supplementary Table 1**). Additionally, we coded different species into 3 categories based on their CR (CR=0, $0<CR\leq450$, CR>450) and plotted the distribution of each predictor in these categories to assess whether there were major differences between them (**Supplementary Fig. 1**). We chose to retain MT and T2T. MT was retained because of its large correlation with CR. T2T was retained for its positive correlation with CR, but also because it contained information about both TYSA and THSA. Because T2T incorporated both TYSA and THSA and because each had a relatively low correlation with CR on their own, those factors were dropped from our set of predictors.

Unfortunately, a robust phylogeny for use in controlling for phylogenetic dependence between data is not available. However, recent advances in our understanding of tiger moth relationships allows for grouping species into certain monophyletic clades (Zaspel et al., 2014; Zenker et al., 2016). We defined the monophyletic clades for our specimens from these studies (**Supplementary Table 2**). Members of each clade are relatively similar morphologically and, along with the known phylogenetic relationships, classification of species into these clades is not difficult in most cases.

To assess the phylogenetic dependence between data to the extent currently possible, we examined the linear relationships between each predictor by the clade to which they belong (**Supplementary Fig. 2**). We found that the relationship between CR and MT was positive within clades, but the slope and possibly the intercept of the relationship may differ between clades. This prompted us to include models with an interaction term between MT and CLADE. Hereafter, we use "CLADE" to refer to the categorical variable modeled in our analyses. We did not find this effect between CR and T2T, therefore we did not examine models with an interaction term between CLADE and T2T.

Our final model set included 7 models. We used Akaike's Information Criterion corrected for small sample size (AICc) to rank and select the best model as implemented in the aictab function of the AICcmodavg package in R (Burnham and Anderson, 2002; Mazerolle, 2016). Models less than 2 ΔAICc units from the "top model" (lowest AICc value) were considered to be of similar support, while models greater than 9-11 ΔAICc units from the top model have relatively low support (Burnham et al., 2010). The Eupseudsomoid clade contains the only tiger moth confirmed via experimentation to jam bat echolocation (Corcoran et al., 2009; Corcoran et al., 2012). Because this is a strategy thought to be reliant on high CR, we coded the factor levels of CLADE such that the significance testing between predictors and CLADE would be relative to this "jamming clade" (Corcoran et al., 2011). The results of AICc model ranking returned two top models of differing complexity from which we infer our results (**Supplementary Table 3**).

Checking Model Assumptions

We determined our final model met the assumptions of linear regression by confirming the mean of the residuals was equal to zero, by visually checking for homoscedasticity of the residuals and normality using the plot command in base R, checking for the absence of autocorrelation with Durbin-Watson test implemented from the lawtest package in R (DW = 1.87, p = 0.24), and by ensuring that the residuals were uncorrelated with the predictors using cor.test function from base R (Gastwirth et al., 2017; R Core Team, 2016). Tables were prepared in LaTeX using the xtable and texreg libraries within R (Dahl, 2016; Leifeld, 2013).

Results

Literature Review

We found 89 articles, master's theses, dissertations, or posters authored or coauthored by 83 unique researchers between 1832 and early 2017 that included information related to the sound production and/or tymbal morphology of tiger moths (**Supplementary Table 5**). Of these accounts, 24 (27%) related to tymbal morphology, 38 (42%) related to sound production, and 28 (31%) contained information about both. We found records for 690 species in 253 genera. Of the approximately 11,000 species and 1,500 genera of tiger moth this amounts to roughly 6% and 17% coverage respectively (Watson and Goodger 1986).

Going forward, we report the highest quality category of tymbal morphology observed among all species within a given genus when tallying results at the generic level. We found that the tymbal morphology of 190 species (28%) and 44 genera (17%) were not characterized. Of the remainder, 181 species (26%) and 76 genera (30%) were

scored as presence/absence, 67 species (10%) and 22 genera (9%) were reported in qualitative categories, 207 species (30%) and 76 genera (30%) included actual counts, while 45 species (7%) and 35 genera (14%) had some kind of image associated with their accounts.

Among the 690 species 215 (31%) were noted to produce sound, 163 (24%) were not found to produce sound, 15 (2%) species were composed of accounts which came to differing conclusions, and 297 (43%) have not been tested. Among our 253 genera 65 (26%) were noted to produce sound, 54 (21%) were not found to produce sound, 37 (15%) contained species accounts that both did and did not produce sound, and 97 (38%) were untested. If untested species are removed, 215 (55%) species produced sound and 163 (41%) did not, with the remainder having conflicting reports. When untested genera are removed, 65 (42%) genera produced sound and 54 (35%) did not, with the remainder having conflicting reports.

Of the 690 species in 253 genera, only 90 species (9%) and 52 genera (21%) have had their sounds recorded using acoustic equipment. Of these, 32 species (36%) and 15 genera (29%) have not had their tymbal morphology characterized. Of the remainder, 14 species (16%) and 7 genera (13%) were scored as presence/absence, 1 species (1%) and 0 genera (0%) were reported in qualitative categories, 29 species (32%) and 20 genera (38%) included actual counts, while 14 species (16%) and 10 genera (19%) were imaged in some way.

The sounds from these 90 species and 52 genera were not elicited in the same manner. Only 62 species (69%) and 44 genera (%) had their sounds elicited using both simulated bat echolocation and tactile stimulation while 6 species (7%) and 4 genera

(8%) were assayed using simulated bat echolocation alone, and 21 species (23%) and 12 genera (23%) were assayed using tactile stimuli alone.

Both tymbal morphology and sound production were available for the same species in only 204 (30%) of cases and in 114 (45%) of genera. We found that the proportion of moth species that produced sound and had microtymbals was significantly greater than moth species that produced sound without microtymbals (Fisher's Exact Test: $p<0.05$, 95% CI: [7.32, 37.6], OR: 15.98).

Of the 690 species and 253 genera, only 13 species (2%) and 11 genera (4%) have been monitored for the presence of acoustic sexual courtship where its presence was demonstrated in 10 species (77%) and 9 genera (82%). Few species' courtships have been acoustically monitored and tymbal morphology was unavailable for 3 (23%) of the species that were examined. Because of this it was difficult to assess the frequency with which sexual dimorphism is associated with acoustic courtship. Of the species which engaged in acoustic courtship, 3 (43%) did not exhibit sexually dimorphic tymbals. All species that did not engage in acoustic courtship did not exhibit sexual dimorphism. There were no species which had sexually dimorphic tymbals and did not engage in acoustic courtship.

Tymbal Morphology and Acoustic Measurements

Data collected in this study are given in **Table 1**. We examined the tymbal morphology and the sounds they produced in response to simulated bat echolocation for 70 species and 38 genera. Of these, 58 species (83%) and 14 genera (37%) had their responses to simulated bat echolocation recorded for the first time. This nearly doubles

the number of existing anti-bat recordings of tiger moths in the literature and grows the number of genera by 31%, bringing the number to 120 species and 59 genera.

62 species (89%) and 21 genera (55%) had their tymbals imaged for the first time. This increases the total number of species with imaged tymbals by 138% to 107 species and the total number of genera increases by 60% to 56 genera. The total number of species with imaged tymbals and recorded anti-bat sounds increases by 563% to 73 species and the number of genera increases by 278% to 36 genera.

For our 70 species, the distribution of click rate, microtymbal counts, and the ratio of tymbal to thorax area we measured is given in **Fig. 2** and descriptive statistics of each are given in **Table 2**.

Predicting Click Rate from Tymbal Morphology

We found two strongly supported models predicting CR from MT, T2T, and CLADE (**Table 3**). Both models explain a large proportion of the variation in CR (Adj. R^2 = 0.80, 0.79). The predictor coefficients and adjusted R^2 are similar for both models. Though the more complex "Model 7" has somewhat lower RMSE compared to "Model 5", this was not a significant difference at the standard cutoff (ANOVA: F=3.4, p=0.07). We built prediction intervals for each model indicating where CR is predicted to lie in 95% of cases for given MT, CLADE, and T2T (Model 5: **Fig. 3**, Model 7: **Fig. 4**). We set predicted values to 0 where CR was predicted to be negative since negative values are not biologically relevant. In order to present the prediction intervals for Model 7 in a 2-D graphic, we plotted two ribbons which represent the minimum (1.8%; dark grey) and

maximum (16.7%; light grey) values for T2T observed in this study to show the extent that the prediction interval would shift depending on the T2T value.

Both models support an intercept that is not significantly different from 0. The results from Model 5 suggest that the slope of the relationship between MT and CR for each CLADE was significantly different from 0, except in the case of the Cisthenoid clade, which was positive, but not significantly greater than 0. However, the sample size for the Cisthenoid clade was very low (n=3).

We constructed a set of level contrasts and applied Model 7 to them to compare the results of each CLADE level to each other CLADE level (**Supplementary Table 4**). This analysis shows that the relationship between MT and CR is significantly greater than 0 in all CLADE levels except Cisthenoid. In comparing the relationship between MT and CR by CLADE level we found that the Eupseudosomoid and Callimorphoid groups had a significantly larger slope than all other CLADE levels, though the Eupseudosomoid group had a larger slope than the Callimorphoid group.

The results indicate that the Eupseudosomoid clade has a significantly greater slope than all other examined clades, suggesting that they produce higher CR for a given number of MT than other clades. Similarly, the Callimorphoid clade appears to be producing a higher CR for a given number of MT compared to all other clades excluding the Eupseudosomoid group. The remaining CLADE levels could not be distinguished as having slopes significantly larger or smaller than any other clade when compared against each other.

We compared CR versus T2T between sound-producing moths without tymbals (n=5) and silent moths without tymbals (n=10) and found no significant difference between means (T-test: t=-0.87, df=5.35, p=0.4), though sample sizes were low. So, mean T2T does not seem to be significantly different between silent and clicking moths which lack microtymbals.

Discussion

In both of our models, MT and CLADE were critical and significant factors for predicting CR. In Model 7, T2T also played a significant, albeit weaker role when compared to MT and CLADE. Because Model 7 did not account for a significantly larger proportion of variance in CR, we prefer Model 5 as it requires measuring only a single aspect of tymbal morphology whereas Model 7 requires three.

The Eupseudosomoids had a significantly larger slope relating CR to MT, indicating that some other factor shared by members of this clade augments the relationship between CR and MT in a positive way that is larger than the effect in other clades (**Fig. 2; Supplementary Fig. 2**). Similarly, the Callimorphoid clade exhibits this same significantly larger and positive "Clade effect" relative to all other clades excluding the Eupseudosomoids. The high duty-cycle sonar-jamming strategy is highly effective at protecting moths from bat predation (Corcoran and Conner, 2012). Therefore, we believe the most likely scenario is that these lineages have evolved other shared mechanisms in addition to the number of microtymbals such as a higher degree of asynchrony between contralateral and ipsalateral tymbal activation, faster half modulation cycles, lower delays between half modulation cycles (*i.e.*, shorter intersilent intervals), lower delays between subsequent full modulation cycles (*i.e.*, shorter inter-cycle intervals), a higher degree to

which the tymbal musculature contracts in each active half modulation cycle, or other factors.

There was only one lineage not found to have a slope significantly different from 0. We believe the most likely explanation for this is the low sample size (n=3) within the Cisthenoid clade. Interestingly, the Cisthenoids were the only members of the lichen-feeding Lithosiini tribe included in our analysis and so perhaps this could be indicative of something related to tribal differences between the Lithosiini and Arctiini. An additional explanation for the Cisthenoid slope coefficient might be that evolving larger MT could allow for less frequent activation while producing the same effective click rate, thereby lowering the metabolic cost of sound production. Under this hypothesis, we would expect species to be under positive selection for larger MT, but not necessarily exhibit a proportional increase in CR in an effort to conserve metabolic resources. However, this hypothesis is not supported by recent experimental evidence from *Bertholdia trigona*, a sonar-jamming species producing many modulation cycles per second, which demonstrated that clicking at high rates has little, if any, significant metabolic cost compared to those of flight (Corcoran and Woods, 2015). However, Cisthenoids measured in this study had a T2T about 3 times higher than that of *B. trigona*, so perhaps the costs of sound production are proportionally higher for them.

It is not yet clear how to infer whether species without microtymbals will produce clicks from tymbal morphology alone. We had hypothesized that silent species would be less likely to have a large T2T, however we did not find support for this hypothesis. Another factor that could be measured in the future is the degree to which the surface of the tymbal organ is scaled. We have observed that silent species tend to have their tymbal

organs completely covered in scales, whereas sound producing species often have fewer, less densely spaced scales. The thickness of the tymbal surface could also be measured, with the expectation that functional tymbals would need to be pliable and thus thinner than non-functional tymbals. This would require a more time intensive preparation of each specimen and necessitate the destruction of the tymbal however.

Likewise, it would be useful to determine whether any factors can explain why some species with microtymbals do not produce sound. It is possible that these represent false negative responses to our simulated bat attacks. We have observed that certain species are more sensitive to the artificial recording conditions and produce sound less readily. It is also possible that some of these species have evolved hearing or pulse interval sensitivities that differ from those produced by *E. fuscus*. An additional possibility is that the tymbal organ in these species is used exclusively for acoustic courtship. However, after examining the tymbal morphology of silent moths with a positive number of microtymbals we discovered that many had either very shallow or irregularly spaced microtymbals which we interpret as a potentially low or non-functioning vestigial state.

We found a number of species exhibit both major and minor microtymbal grooves, which may lead to inflations or reductions in the reported microtymbal counts between studies. We suggest that, until the minor grooves are shown to add to acoustic complexity, they should not be counted as true “microtymbals”. Instead, only the major grooves, those deep depressions in the surface of the microtymbal usually accompanied by a singular scale socket, should be considered as contributing to the number of discrete clicks produced by a tymbal.

We do not believe that the intraspecific differences in detected sound production discovered in our literature review necessarily constitute evidence for wide-spread intraspecific variation in acoustic responses. Instead, we believe it is more likely accounted for by differences in how the clicks were elicited, the observation or recording methodology, or possibly other factors such as moth age.

After many trials with moths it seems clear that not all individuals of a given species, even within the same sex, respond to stimulation the same or at all. It is unclear precisely what determines whether a moth will respond. It could be a real difference or it may be due to our methodology which necessitates restraining the wings. Re-examining individuals of "silent" species on their first night post-eclosion might be fruitful. It could also be that some other factor was not sufficiently replicated in our experimental design. For instance, we have found that tiger moths respond to simulated bat echolocation much more frequently when flight is better simulated by blowing air on them with a small fan such that their legs are held close to the body as they would be in natural flight. All tiger moths we have assayed for anti-bat responses fail to respond to bat ultrasound while sitting on a substrate. Thus, we emphasize that the interpretation of reports indicating a lack of sound production among tiger moths should bear in mind the context and methods used to elicit their response, the sex and number of individuals used to make that determination, and other factors that may have inhibited their normal sound production.

We did not examine sound-production in the context of sexual courtship, however it seems unlikely that we would be able to predict this function from morphology alone except in certain cases. Though reports of acoustic courtship among tiger moths are relatively sparse, our analysis of the available data suggest that sexual dimorphism in

tymbal morphology is sufficient, but not necessary evidence for acoustic courtship. Most tiger moth species studied to date which use sound in courtship appear to modify the pattern and timing of tymbal activation to produce sexually dimorphic signals using essentially the same tymbal morphology (Sanderford et al., 1998). It is unclear how the courtship sounds of conspecifics elicit an acoustic response that is markedly different from the typified responses used against bat predators or in response to tactile stimulation.

The results of this study are a significant first step toward predicting the sound production of tiger moth species from morphology alone, and perhaps even the function of those sounds. However, before the function of these sounds can possible be inferred, two key experiments must be conducted. We chose to measure the amount of sound produced by moths in terms of click rate instead of the recent trend of reporting duty-cycle (see Methods). It is still unclear which of these is more informative about the sonar-jamming qualities of tiger moth sounds. Experiments that expand on past studies by varying the number and duration of clicks presented within the jamming window are needed to determine which measure of sound production best predicts a species' jamming capabilities. If duty-cycle is found to be more indicative of a sonar-jamming function, we would need to additionally model what aspects of tymbal morphology determine the duration of each click, as duty-cycle is defined as click rate multiplied by the duration of each click.

The last remaining hurdle would be determining which click rates or duty-cycles produce a sonar-jamming effect and which do not. It has been suggested that there could be a "duty-cycle threshold" (Corcoran et al., 2010). When moth sounds are produced at a

duty-cycle above this threshold value it is hypothesized that they will occur with sufficient frequency to reliably cause a sonar jamming effect. When these sounds are produced at a rate below this threshold value they are hypothesized to occur too infrequently to reliably disrupt a critical proportion of bat echoes, instead functioning in acoustic aposematic signaling. Empirical experiments varying the click rate (or duty-cycle) are needed to determine if this threshold exists, and if so, what the threshold value is.

By combining predicted click rate and a click rate threshold for sonar jamming we can construct strong hypotheses which assign species to particular acoustic anti-bat strategies (aposematic signaling versus sonar-jamming) with a certain level of confidence. These hypotheses could be tested using previously demonstrated methods (Corcoran and Conner, 2012; Dowdy and Conner, 2016) and would also aid us in understanding the evolutionary patterns of sound production in tiger moths (*e.g.*, have multiple lineages of tiger moths independently converged on a sonar-jamming anti-bat strategy?).

The models presented in this study should be viewed as well-supported hypotheses, but their predictive powers should be verified using an independent dataset of moth sounds and tymbal morphology, preferably sampled from species and genera not included in this survey to ensure that the model is capable of accurately predicting the acoustic characteristics from a diverse array of tiger moths.

While our models explain a large amount of variation in CR they could be further improved. By adding data from more species, particularly from genera that have not yet been included, our prediction intervals should perform better for a more varied

assortment of species. Data from individuals within clades that were not represented in this analysis should be added so that CR can be predicted reliably for those individuals. In addition, more accurate and robust phylogenies that include a greater diversity of taxa would allow us to better control and model phylogenetic dependence. We could also incorporate additional predictor variables to better understand and account for the underlying sources of variation in click rate. Furthermore, this approach could easily be applied to other important aspects of tiger moth sounds. For example, this approach could be used to study which tymbal features account for the high degree of variation observed between species in the dominant frequency, intensity, or duration of clicks.

We have not yet assessed variation in tymbal morphology and sound production for species with large geographic ranges or species that occur at different times of the year. We expect that as selective pressures vary by geographic region tymbal morphology and acoustic characteristics will also vary, possibly even within species. This may be particularly evident at extreme latitudes, as bat species richness, in general, is greatest near the equator and decreases at higher latitudes (Ramos Pereira and Palmeirim, 2013). Fullard was the first to address how tiger moth sounds varied between species that were active at different times of the year (Fullard, 1977b). He found that one genus, *Phragmatobia*, contained two species which overlapped in geographic range, but differed in the time of year in which they were active. The species which was active early in the season was silent, while the species which was active concurrently with bat predators produced sound in response to tactile stimulation. To our knowledge, no one has yet examined the existence of this phenomenon within a species that exhibits broad active periods with multiple broods. Intraspecific variation in sound production might then

relate to differential bat predation between broods that are active at different times of the year.

While intraspecific variation in sound production is plausible, there is also growing evidence of cryptic species within the Arctiinae and so variation described as intraspecific could actually be attributable to interspecific variation (Janzen and Hallwachs, 2016; Zenker et al., 2016). Unfortunately, explaining disagreement between studies or attributing sources of variation to either intra- or interspecific variation is impossible without some physical vouchering. The majority of records we included in our literature review, particularly those records that related to sound production, did not include high-quality images or information about the current location of the specimens they tested. This is problematic because it is difficult to assess the reliability of the species identifications and, by extension, our confidence in the data attributed to those species. We believe that efforts to voucher more specimens used in behavioral studies would also benefit the scientific community, not only so that positive species identifications can be made when needed, but also so that those specimens might be given renewed usefulness in future studies of phenomenon perhaps not yet considered by current researchers.

Natural history collections are invaluable sources of data for disciplines as diverse as biogeography, ecology, genetics, and systematics. Yet even recent reviews of their potential usefulness overlook their possible applications to the study of behavior (Lane, 1996; Holmes et al., 2016; McLean et al., 2016). We believe this study is a good example of how even preserved specimens can provide useful information about the behaviors these specimens may have exhibited in life. We expect that investigations of other animal

behaviors could also benefit from collections-based research to lead to transformative insights about the diversity and distribution of behaviors on large spatial or temporal scales.

In the majority of cases, characterization of tymbal morphology has not been included in species descriptions within the Arctiinae. Some recent taxonomic works have given basic accounts of the tymbal organ in descriptions of new species and we encourage the continuation of this practice and suggest future authors report a count of the number of microtymbals exhibited by the species they describe (Vincent et al., 2014). While it is unclear whether morphological features of the tymbal organ reflect systematic information, including descriptions of these features could yield insights into the behavioral ecology of the organism that would be useful for understanding the evolution of sound production within the Arctiinae.

Conclusion

More and more we are discovering that among moths, the Arctiinae are not alone in utilizing tymbals for sound production. A number of major moth lineages have convergently evolved tymbal-like organs for sound production for use in courtship or defense (*e.g.*, Geometridae (Corcoran and Hristov, 2014); Nolidae (Skals and Surlykke, 1999); Lymantriinae (Dall'Asta, 1988); Noctuidae (Heller and Achmann, 1993); Pyralidae (Spangler et al., 1984); Crambidae (Heller and Krahe, 1994)). It is likely that the methods from this study will be generally applicable to other moth groups since their tymbals share the same basic structure – paired, air-filled sacs with striated regions – with the tymbals of the tiger moths and function in a similar manner.

While the work performed over the last 185 years has had a huge impact on our knowledge of the behavior ecology and sound production within the Arctiinae, we are still missing information for a significant number of species (~93%) and genera (~80%). Notably, we still lack published information regarding the sound production of entire subtribes (*e.g.*, Phryganopterygini). There is surely much left to be learned about sound production amongst tiger moths, particularly as it relates to acoustic sexual courtship.

The models presented here cannot and should not replace the direct measurement of anti-bat sounds. However, directly measuring the sounds of large numbers of species would require significant resources and time. In addition, while tiger moths occur worldwide, their diversity is highest in tropical regions where the probability of significant biodiversity loss is very high (Brooks et al., 2002). As the effects of deforestation, global climate change, and other sources of biodiversity loss continue largely unabated, it seems inevitable that the sounds of some species will go unrecorded and unheard by humans (Butchart et al., 2010). We believe our models can be used to great effect as a complement to direct measurements of sound production in order to quickly and broadly expand our understanding of the general trends in acoustic qualities of tiger moth sounds and the anti-predator strategies they employ. Finally, these results may also have some practical engineering applications. By understanding the factors that allow these animals to produce intense sounds (up to 100dBSPL @ 5 cm) in an extremely wide range of frequencies (10kHz - 200kHz) using a compact transducer (~1 mm^2), we may learn how to produce smaller, less expensive ultrasonic speakers with improved acoustic properties.

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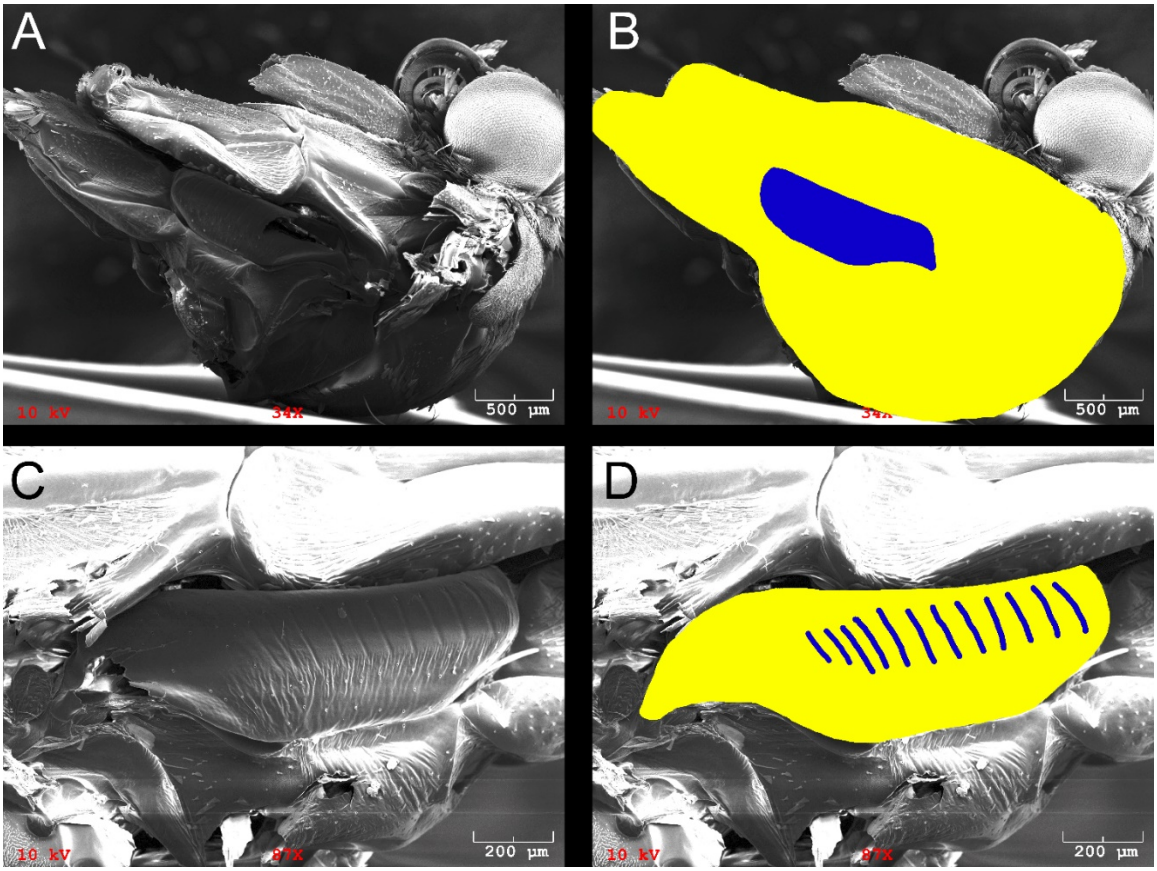
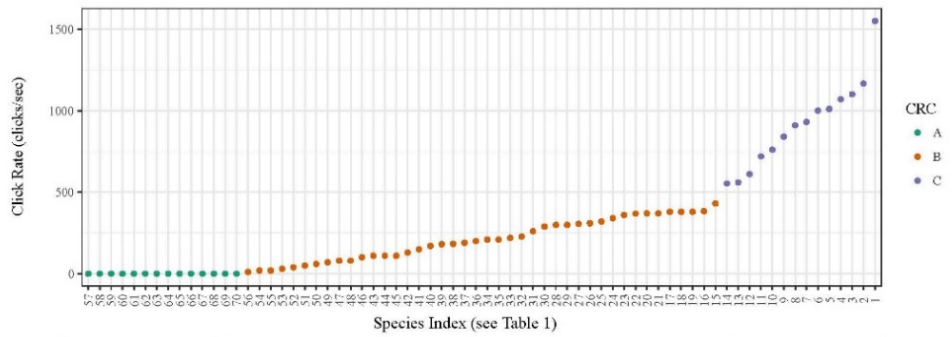
Figure 1. Scanning electron micrographs of *Cisthene martini*. A) Thorax with tymbal. B) Body with tymbal outlined as Adobe Photoshop layers for measuring surface area. C) Zoomed image of tymbal. D) Zoomed image of tymbal outlined with microtymbals highlighted as Adobe Photoshop layers to facilitate counting. Scale bars in each image.

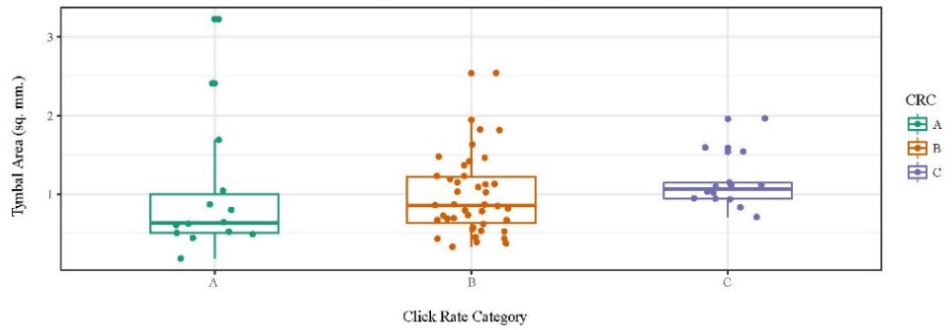


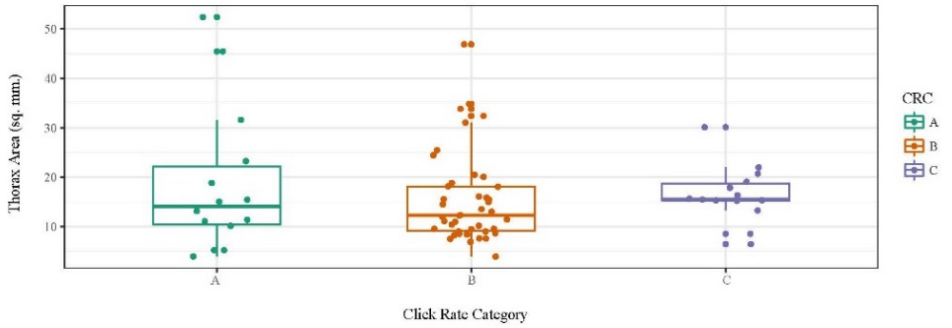
Supplementary Table 1. Correlation matrix.

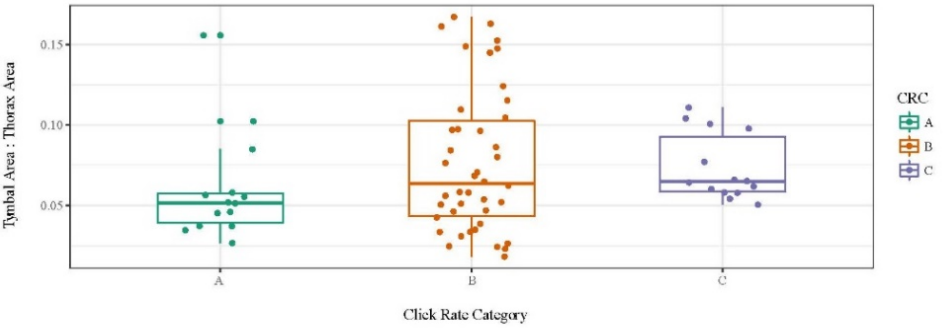
	CR	MT	TYSA	THSA	T2T
CR	1.00	-	-	-	-
MT	0.66	1.00	-	-	-
TYSA	0.13	0.09	1.00	-	-
THSA	-0.05	-0.04	0.51	1.00	-
T2T	0.12	0.14	0.38	-0.45	1.00

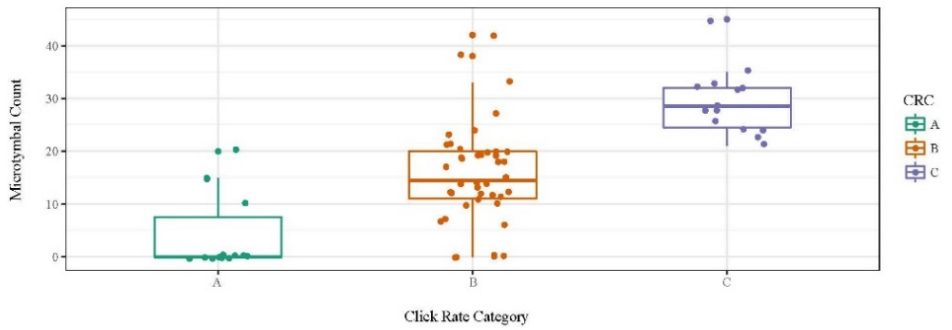
Supplementary Figure 1. Distribution of each predictor within three click rate categories (CRC) (Continued on Next Page). CRC categories defined as: A (CR = 0), B ($0 < CR \leq 450$), C ($CR > 450$).


Click Rate (clicks/sec)
1500
1000
500
0
Species Index (see Table 1)
CRC
A
B
C


Tymbal Area (sq. mm.)
3
2
1
A
B
C
Click Rate Category
CRC
A
B
C


Thorax Area (sq. mm.)
50
40
30
20
10
A
B
C
Click Rate Category
CRC
A
B
C

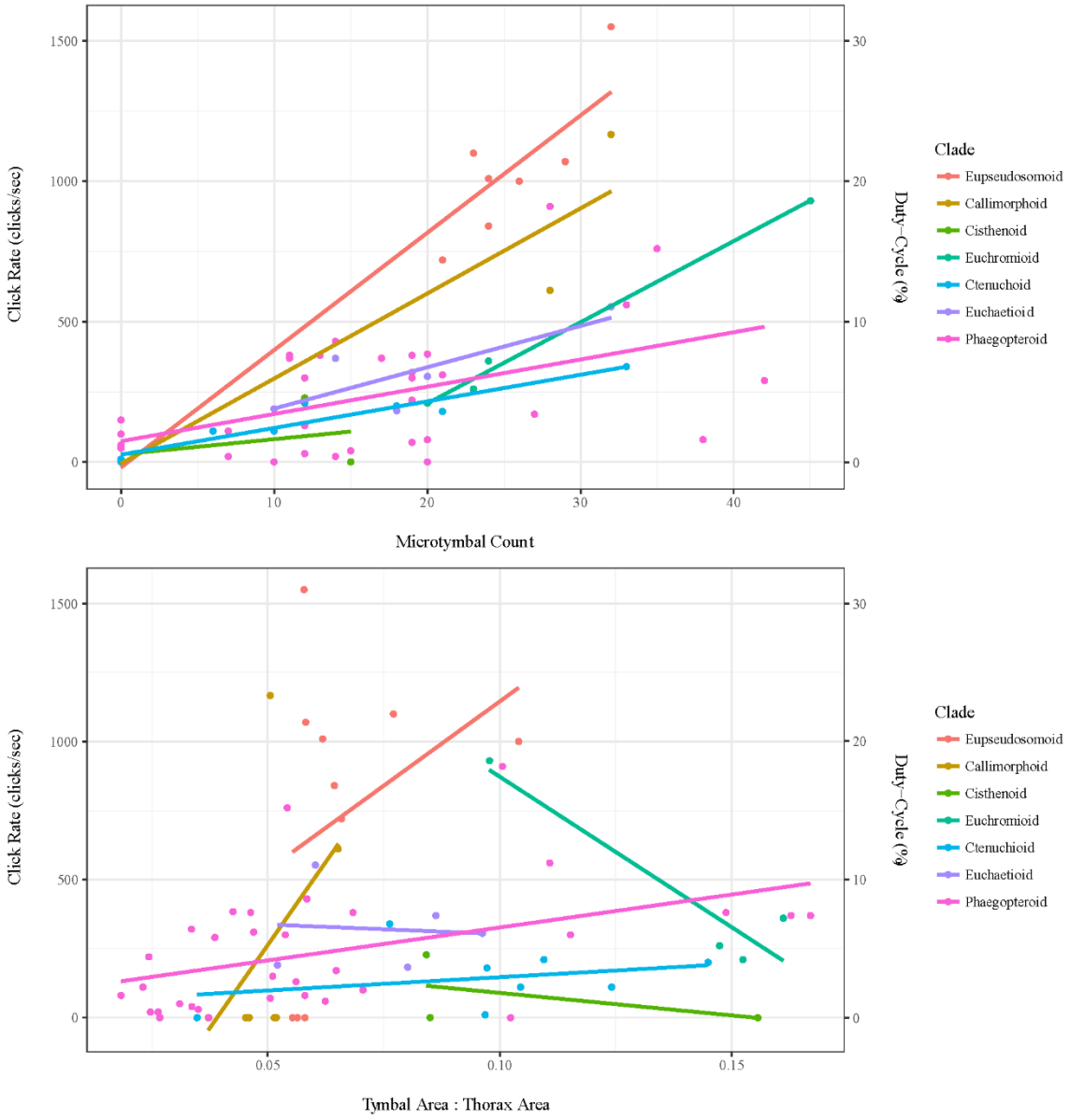

Tymbal Area : Thorax Area
0.15
0.10
0.05
A
B
C
Click Rate Category
CRC
A
B
C


Microtymbal Count
40
30
20
10
0
A
B
C
Click Rate Category
CRC
A
B
C

Supplementary Table 2. Monophyletic clades used in this study. Clades defined by the node joining the two taxa listed and all its descendants. See phylogeny in Zenker et al., 2016 for comparison.

Clade Name	Branch 1	Branch 2
Eupseudosomoid	Cissura decora	Ordishia rutilus
Callimorphoid	Utetheisa lotrix	Virbia fragilis
Euchaetioid	Pagara simplex	Agaraea semivitrea
Euchromioid	Macrocneme sp.	Dycladia lucetius
Phaegopteroid	Anaxita sp. 1	Pachydota affinis
Ctenuchoid	Neotrichura nigripes	Ctenucha virginica
Cisthenoid	Praepiella sesapina	Ardonea tenebrosa

Supplementary Figure 2. Plots of MT and T2T against CR.



Supplementary Table 3. Model Comparisons.

	Model	K	AICc	Delta AICc	AICc weight	log-Likelihood
7	CR ~ MT + T2T + MT:CLADE	10.00	922.75	0.00	0.63	-449.51
5	CR ~ MT:CLADE	9.00	923.84	1.09	0.37	-451.42
8	CR ~ MT * CLADE + T2T	16.00	932.08	9.33	0.01	-444.91
6	CR ~ MT * CLADE	15.00	937.19	14.44	0.00	-449.15
2	CR ~ MT	3.00	984.81	62.06	0.00	-489.22
4	CR ~ MT + T2T	4.00	986.94	64.19	0.00	-489.16
1	Intercept Only (Null)	2.00	1022.19	99.44	0.00	-509.01
3	CR ~ T2T	3.00	1023.32	100.56	0.00	-508.48

Table 1. Data measured in this study. Click rate is measured in clicks per second. Areas are measured in square millimeters.

Index	Genus_species	Clade	Click Rate[1]	Microtymbal Count	Tymbal Area[2]	Thorax Area[2]	Tymbal:Thorax Area
1	Bertholdia_trigona	Eupseudosomoid	1550	32	1.10	19.03	0.06
2	Haploa_clymene	Callimorphoid	1170	32	1.11	21.98	0.05
3	Bertholdia_partita	Eupseudosomoid	1100	23	1.02	13.23	0.08
4	Idalus_pichensis	Eupseudosomoid	1070	29	0.95	16.33	0.06
5	Melese_amastris	Eupseudosomoid	1010	24	0.94	15.20	0.06
6	Melese_intensa	Eupseudosomoid	1000	26	1.59	15.28	0.10
7	Cosmosoma_nr_centralis	Euchromioid	930	45	0.83	8.49	0.10
8	Carales_arizonensis	Phaegopteroid	910	28	1.54	15.31	0.10
9	Bertholdia_ockendeni	Eupseudosomoid	840	24	1.15	17.87	0.06
10	Halysidota_melaleuca	Phaegopteroid	760	35	1.12	20.67	0.05
11	Bertholdia_sp	Eupseudosomoid	720	21	1.03	15.63	0.07
12	Pyrrharctia_isabella	Callimorphoid	610	28	1.96	30.07	0.07
13	Ischnocampa_nr_discopunta	Phaegopteroid	560	33	0.71	6.41	0.11
14	Euerythra_phasma	Euchaetioid	550	32	0.93	15.44	0.06
15	Pachydota_rosenbergi	Phaegopteroid	430	14	1.81	31.00	0.06
16	Pseudohemihyalea_edwardsii	Phaegopteroid	380	20	1.48	34.82	0.04
17	Ischnocampa_nubilosa	Phaegopteroid	380	13	1.23	8.27	0.15
18	Leucanopsis_sp_8	Phaegopteroid	380	19	1.13	24.41	0.05
19	Phaegoptera_decrepidoides	Phaegopteroid	380	11	1.02	14.92	0.07
20	Cycnia_tenera	Euchaetioid	370	14	0.82	9.51	0.09
21	Ischnocampa_nr nubilosa	Phaegopteroid	370	11	1.15	6.89	0.17
22	Ischnocampa_nr_obscurata	Phaegopteroid	370	17	1.46	8.97	0.16
23	Phaeo_aquiguttata	Euchromioid	360	24	1.63	10.12	0.16
24	Eucereon_nr_coeruleocaput	Ctenuchoid	340	33	1.03	13.50	0.08
25	Praemastus_minerva	Phaegopteroid	320	19	0.52	15.51	0.03
26	Pelochyta_gandolfii	Phaegopteroid	310	21	0.85	18.10	0.05
27	Pygarctia_roseicapitis	Euchaetioid	310	20	0.72	7.48	0.10
28	Lophocampa_sp	Phaegopteroid	300	19	0.45	8.37	0.05
29	Ischnocampa_nr hemihyala	Phaegopteroid	300	12	0.87	7.55	0.12
30	Pelochyta_sp_indet	Phaegopteroid	290	42	0.62	16.06	0.04
31	Cosmosoma_vespoides	Euchromioid	260	23	1.12	7.60	0.15
32	Cisthene_martini	Cisthenoid	230	12	0.33	3.92	0.08
33	Praemastus_minerva_watkinsi	Phaegopteroid	220	19	0.44	18.05	0.02
34	Mesothen_nomia	Euchromioid	210	20	1.37	8.99	0.15
35	Correbia_bricenoi	Ctenuchoid	210	12	1.42	12.97	0.11
36	Eucereon_coeruleocaput	Ctenuchoid	200	18	1.23	8.49	0.14
37	Euchaetes_antica	Euchaetioid	190	10	0.57	10.94	0.05
38	Euchaetes_egle	Euchaetioid	180	18	0.69	8.61	0.08
39	Eucereon_rogersi	Ctenuchoid	180	21	1.95	20.05	0.10
40	Leucanopsis_bactris	Phaegopteroid	170	27	0.79	12.20	0.06
41	Lophocampa_nr_endrolepia	Phaegopteroid	150	0	0.53	10.38	0.05
42	Leucanopsis_sp	Phaegopteroid	130	12	0.87	15.51	0.06
43	Ctenucha_venosa	Ctenuchoid	110	6	2.54	20.47	0.12
44	Amastus_coccinator	Phaegopteroid	110	7	0.78	33.81	0.02
45	Eucereon_pseudocasca	Ctenuchoid	110	10	1.19	11.39	0.10
46	Ischnocampa_nr_lugubris	Phaegopteroid	100	0	0.67	9.50	0.07
47	Amastus_thermidora	Phaegopteroid	80	20	0.86	46.85	0.02
48	Onythes_melanchra	Phaegopteroid	80	38	0.55	9.48	0.06
49	Leucanopsis_nr_oruba	Phaegopteroid	70	19	0.73	14.44	0.05
50	Aemilia_rubriplaga	Phaegopteroid	60	0	0.69	11.06	0.06
51	Lophocampa_distincta	Phaegopteroid	50	0	0.37	11.94	0.03
52	Praemastus_watkinsi	Phaegopteroid	40	15	1.09	32.40	0.03
53	Amastus_vitripennis	Phaegopteroid	30	12	0.43	12.29	0.03
54	Amastus_erganoides	Phaegopteroid	20	14	0.67	25.42	0.03
55	Hyalarctia_sp	Phaegopteroid	20	7	0.39	15.80	0.02
56	Eucereon_lineatum	Ctenuchoid	10	0	1.82	18.79	0.10
57	Eudesmia_arida	Cisthenoid	0	0	0.44	5.18	0.08
58	Hypercompe_scribonia	Callimorphoid	0	0	2.41	52.38	0.05
59	Hyphantria_cunea	Callimorphoid	0	0	0.50	11.05	0.05
60	Amastus_rosenbergi	Phaegopteroid	0	15	0.62	23.22	0.03
61	Palaeomolis_nr_palmeri	Callimorphoid	0	0	0.52	10.12	0.05
62	Josioides_sp1	Cisthenoid	0	15	0.61	3.92	0.16
63	Palaeomolis_rothschildi	Callimorphoid	0	0	0.80	15.43	0.05
64	Hypercompe_robusta	Callimorphoid	0	0	1.69	45.46	0.04
65	Symphlebia_apud_juvenis	Eupseudosomoid	0	0	0.64	11.35	0.06
66	Pelochyta_nr_deceptura	Phaegopteroid	0	10	0.49	13.14	0.04
67	Pachydota_nervosa	Phaegopteroid	0	20	3.23	31.57	0.10
68	Neonerita_syrissa	Eupseudosomoid	0	0	1.04	18.79	0.06
69	Symphlebia_juvenis	Eupseudosomoid	0	0	0.87	15.00	0.06
70	Holophaea_endoleuca	Ctenuchoid	0	0	0.18	5.18	0.03

Figure 2. Descriptive statistics of CR, MT, and T2T.

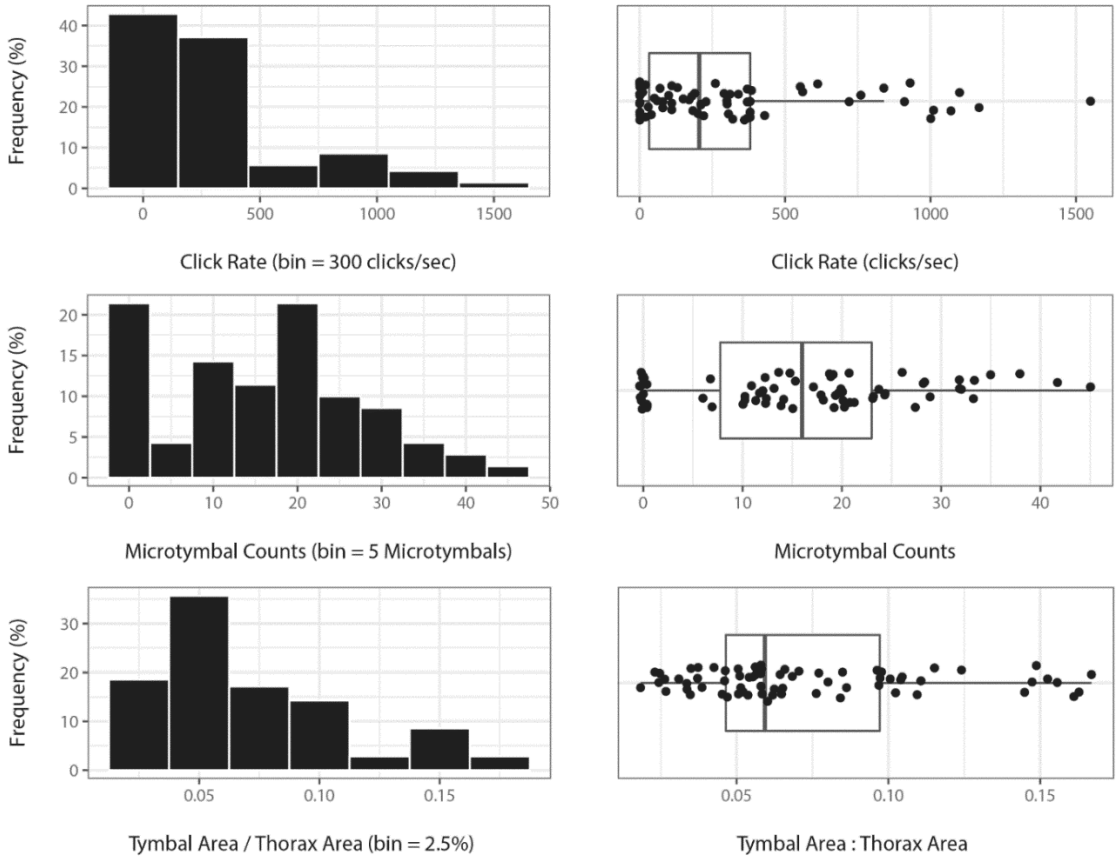


Table 2. Additional descriptive statistics of CR, MT, and T2T.

	Mean	Std. Dev.	Std. Err.	Upper 95 CI	Lower 95 CI	Median	Min	Max	Unit
Click Rate	308.43	350.65	82.15	390.57	226.28	205.00	0.00	1550.00	$Clicks*sec^{-1}$
Microtymbal Count	16.01	11.58	2.71	18.73	13.30	16.00	0.00	45.00	Count
Tymbal:Thorax Area	0.07	0.04	0.01	0.08	0.06	0.06	0.02	0.17	Ratio

Figure 3. Model 5 with 95% Prediction Intervals. For a given MT and CLADE, CR is expected to fall within these intervals in 95% of cases.

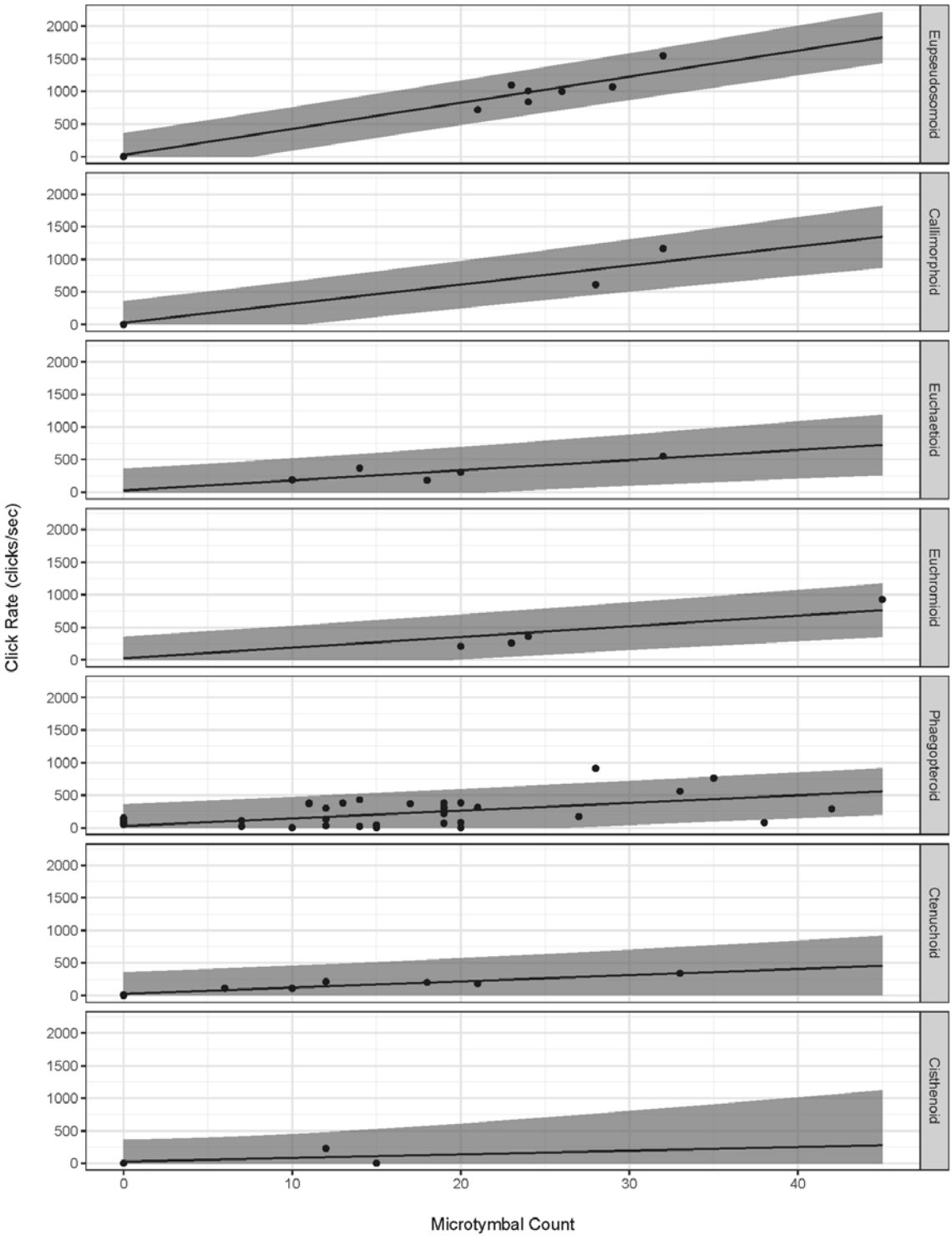
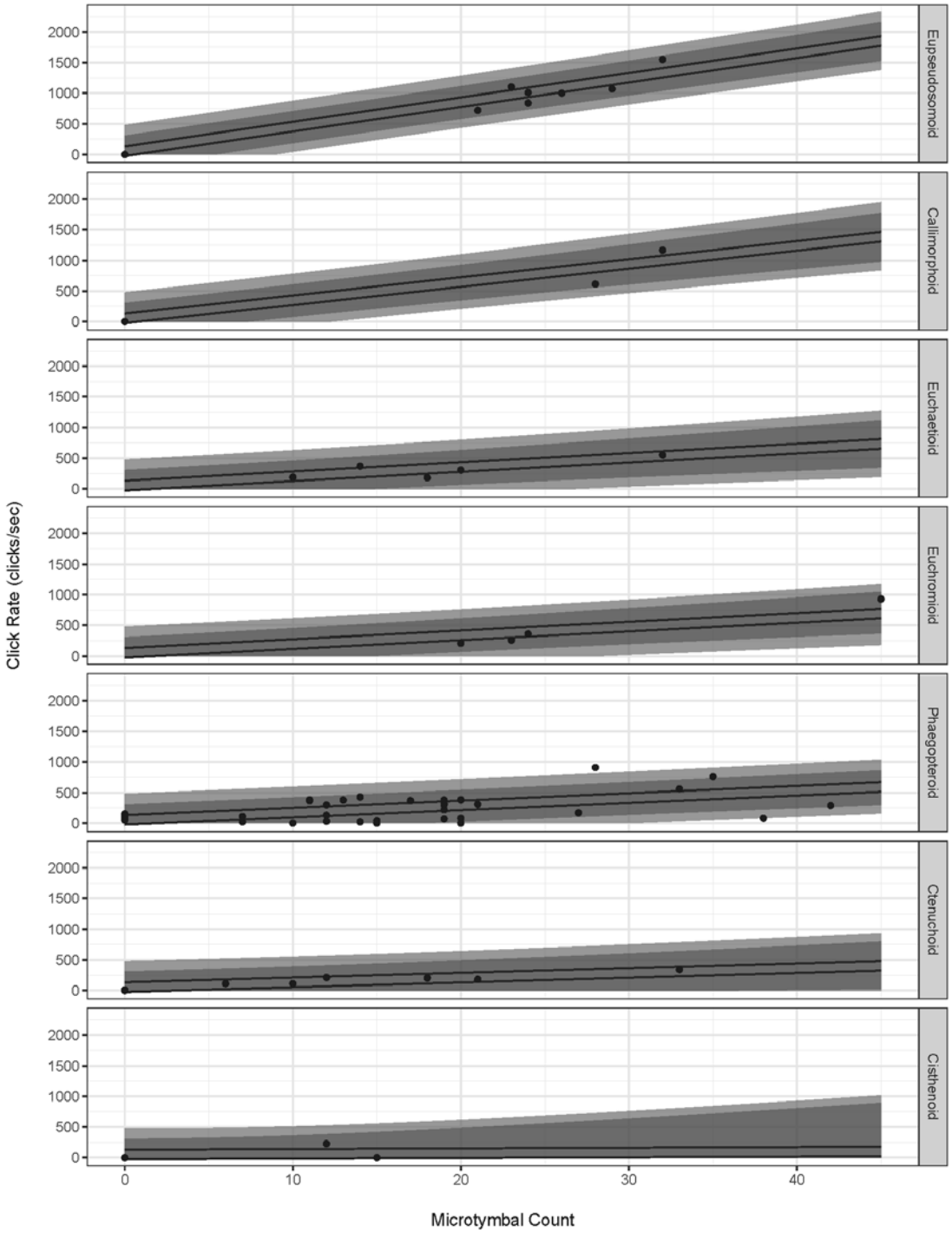


Figure 4. Model 7 with 95% Prediction Intervals. For a given MT, CLADE and T2T, CR is expected to fall within these intervals in 95% of cases. T2T shifts this prediction interval up or down depending on its value. To show this in a 2D figure, we plot the two most extreme intervals from our measured T2T values.



Supplementary Table 4. Contrast matrix for Model 7. Significant differences in regression coefficients between CLADE levels with standard errors in parentheses.

(Intercept)	−43.47	−43.47	−43.47	−43.47	−43.47	−43.47	−43.47
	(49.93)	(49.93)	(49.93)	(49.93)	(49.93)	(49.93)	(49.93)
MT	39.92***	29.56***	15.09***	14.18***	11.92***	7.69*	1.05
	(2.66)	(3.91)	(3.85)	(3.12)	(2.03)	(3.92)	(8.99)
T2T	1053.52+	1053.52+	1053.52+	1053.52+	1053.52+	1053.52+	1053.52+
	(569.90)	(569.90)	(569.90)	(569.90)	(569.90)	(569.90)	(569.90)
MT:Eupseudosomoid		10.36*	24.83***	25.74***	28.00***	32.23***	38.87***
		(4.43)	(4.24)	(3.73)	(2.74)	(4.27)	(9.01)
MT:Callimorphoid	−10.36*		14.47**	15.38**	17.64***	21.87***	28.51**
	(4.43)		(5.18)	(4.80)	(4.02)	(5.24)	(9.56)
MT:Euchaetioid	−24.83***	−14.47**		0.91	3.17	7.40	14.04
	(4.24)	(5.18)		(4.56)	(3.81)	(4.99)	(9.32)
MT:Euchromioid	−25.74***	−15.38**	−0.91		2.26	6.49	13.13
	(3.73)	(4.80)	(4.56)		(3.30)	(4.40)	(8.92)
MT:Phaegopteroid	−28.00***	−17.64***	−3.17	−2.26		4.23	10.87
	(2.74)	(4.02)	(3.81)	(3.30)		(3.88)	(8.84)
MT:Ctenuchoid	−32.23***	−21.87***	−7.40	−6.49	−4.23		6.64
	(4.27)	(5.24)	(4.99)	(4.40)	(3.88)		(9.15)
MT:Cisthenoid	−38.87***	−28.51**	−14.04	−13.13	−10.87	−6.64	
	(9.01)	(9.56)	(9.32)	(8.92)	(8.84)	(9.15)	
R^2	0.82	0.82	0.82	0.82	0.82	0.82	0.82
Adj. R^2	0.79	0.79	0.79	0.79	0.79	0.79	0.79
Num. obs.	70	70	70	70	70	70	70
RMSE	159.41	159.41	159.41	159.41	159.41	159.41	159.41

$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $^{+}p < 0.10$

Supplementary Table 5. Compiled literature review results. See: https://doi.org/10.6084/m9.figshare.4964252

CHAPTER IV

NOVEL USE OF MOTH FLOCCULENT AS A DEFENSE AGAINST BAT PREDATION

Research Article

Novel use of moth flocculent as a defense against bat predation

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Abstract

Males of certain tiger moth (Erebidae: Arctiinae) species within the tribe Euchromiina possess a trait called flocculent. Flocculent is composed of deciduous, modified scales impregnated with toxic compounds sequestered by male moths from their host plants. Flocculent has previously been documented to be involved in sexual courtship and in protecting moths from spider predation. We here document the flocculent emission of *Homoeocera trizona* in response to restraint for the first time and analyze its chemical composition using HPLC-MS/MS and GC-MS methods. We found that the flocculent contains a diverse and highly concentrated array of toxic, plant-derived pyrrolizidine alkaloids. Additionally, we found that the flocculent contains the male tiger moth pheromone hydroxydanaidal. This study is the first to examine interactions between flocculent-bearing tiger moth species with free-flying bat predators. We report that flocculent is released by *H. trizona* when captured by bats, whereupon the moths are released unharmed, effectively warding off their would-be predators.

Keywords: *Homoeocera trizona*, Flocculent, Arctiinae, Anti-predator defense, pyrrolizidine alkaloids

Introduction

Males of certain tiger moths (Erebidae: Arctinae) within the tribe Euchromiina possess a unique trait called flocculent (Blest, 1964). Flocculent is composed of cuticular, deciduous filaments which are stored in either one or two subabdominal pouches (Weller et al., 2000). Flocculent plays a critical role in the courtship of *Cosmosoma myrodora*, where males sequester toxic pyrrolizidine alkaloids (PAs) from plants and allocate some of these toxins to the flocculent. Once the male has found a female he discharges the flocculent, covering the female in a 'nuptial veil' which protects her from spider predation (Conner et al., 2000). Published reports indicate that male *C. myrodora* do not use this defense directly against predators, releasing their flocculent only in courtship (Conner et al., 2000). In contrast, males of some Euchromiine species such as *Homoeocera stictostoma* have been noted to release flocculent under physical restraint allowing males to escape entrapment in spider webs (Müller, 1874; Blest, 1964; Yack et al., 2004). Though bats are a major predator of moths, flocculent has never been demonstrated as an effective post-capture anti-bat defense. In this study, we document the emission of flocculent by *Homoeocera trizona* for the first time and describe its physical structure and chemical composition. We here present the first evidence of a Euchromiine utilizing its unique flocculent defense against bat predators.

Methods

Ethics Statement

Some vertebrates (bats) were captured or handled during these experiments. The methods employed in this study were approved by the Ecuadorian government (Permit

003-15-IC-FAU-DNB/MA issued by the Ecuadorian Ministry of Environment). The methods of this study were approved by the Wake Forest University Institutional Animal Care and Use Committee (protocol #A14-091). This work was performed with permission on private property.

Field Site and Insect Capture Method

Field experiments were conducted at the Yanayacu Biological Station and Center for Creative Studies (YBS) approximately 5 km west of Cosanga, Ecuador. The GPS coordinates of the field site are: 00°36.235' S, 77°52.917' W; elevation: 2,100 m. This location was chosen based on the impressive amount of information available regarding the many moth species present there (Rab-Green et al., 2011). YBS lies on the eastern slopes of the Andes and is comprised of primary forest as well as partially reforested pastures and roadsides.

Insects were collected on station grounds from sheets illuminated with 15 Watt ultraviolet "quantum" lights (Leptraps.com; F15T8QBL). Moths identified as *H. trizona* (Dognin, 1906) were carefully placed individually in 30mL plastic containers and stored for up to 24 hours at ambient temperatures (10-16° C). At our field site, this species rarely came to our lights during the months of July and August 2014, where only 3 specimens were collected. The species was more common during the months of March and April 2015, where 7 specimens were collected.

H. trizona males have a mean forewing length of 1.93±0.02cm (n=3; mean±SE). Forewings are denuded and clear with black wing venation. Hindwings are denuded and have a pearlescent-blue shimmer and black wing venation. The thorax and posterior end of

the abdomen are black, while the anterior segments of the abdomen are bright white providing stark contrast. The flocculent and the subabdominal flocculent pouches are also a conspicuously colored white.

Bat Capture and Housing

Bats were captured using mist nets set up at 17:30 on April 6, 2015 near the exit of a roost on private property. Only bats within the genus *Myotis* were kept for inclusion in this study. Bats that did not belong to *Myotis* were released. Immediately after capture bat health and condition was assessed, including wing condition, parasite load, and the condition of the soft tissues around the nose, mouth, and ears. Each bat's activity level was also assessed and those that were overly lethargic or aggressive were released immediately. In addition, juveniles and lactating females were released immediately upon capture. Unique patches of hair were clipped from the dorsal side of each bat to identify them throughout the study. The bats were placed in pre-labeled, clean cotton bags (30 x 45 cm), as recommended by Kunz and Kurta (1988) and were transported back to their housing area on station grounds.

The captured bats were housed in a custom-built enclosure, measuring approximately 5.5 x 4.5 x 3.0 meters (L x W x H). The roof and sides of the enclosure were covered with sheer netting. Small native trees and logs were potted and placed within the enclosure to enrich the flight space. A single work light (60W) was placed in the enclosure to provide illumination and induce flight in moths which were brought into the enclosure. A small pan filled with water was placed within the enclosure to allow bats constant access to water. The bats were allowed to fly and forage freely throughout the night. At the corners of the enclosure small rags were secured to act as roosting areas.

The bats were captured by hand from their roosting areas within the enclosure at the end of each night. They were given water administered via plastic medicine dropper and hand fed freshly collected moths until satiation to ensure they obtained adequate nutrition for the night. After feeding, the weight of each bat was measured and recorded. The bats were put into groups of 2-3 individuals and then placed into a large sealed sack to sleep during the day. This was done to ensure that the bats would not get too cold while sleeping. If any bats showed signs of stress, sickness, lethargy, weight loss, dehydration, or an unwillingness to fly, eat, or drink at any point they were immediately removed from the study and released at their site of capture. At the conclusion of the study all bats were released at their approximate times and sites of capture.

Experimental Setup

Bats were trained to capture free-flying moths around a work light in a custom enclosure over the course of approximately one week. Initially, moths were continuously added to the enclosure in large numbers such that the bats had a practically unlimited supply of moths to feed on. The number of free-flying moths was gradually reduced and replaced with moths that were tossed into the air by hand around the work light. In the final stages of training, bats fed consistently on moths tossed into the air while continuing to supplement their feeding with the remaining free-flying moths in the enclosure. Once trained to consistently take moths tossed into the air, experimental moths were added to the routine.

Moth Surgical Procedure

Because of the unique defense possessed by *H. trizona* it was impossible to handle the moths without causing immediate flocculent release while the moths were active. To perform the necessary surgeries the moths were first removed from their containers and placed in a freezer on an ice block for no longer than 2-3 minutes until anesthetized. The wings were then folded above the thorax and secured using a hemostat. Surgeries were rapidly performed with the specimen placed on the ice block to ensure the moth would not become active while being handled. Both tympanic membranes were ablated with a pin. The tymbal organs were completely ablated with forceps. Neither procedure caused a significant loss of hemolymph nor flocculent. After the surgery, moths were allowed to recover for a few hours at ambient temperatures in their 30mL cups.

Scanning Electron Microscopy

We used a scanning electron microscope (SEM) (Model: Amray 1810) to image the flocculent of *H. trizona*. Critical point drying was not necessary for these specimens. A small amount of flocculent was removed from the pouch of a specimen and placed on a stub with double-sided carbon tap. The sample was gold coated in a sputter coater (Model: Cressington Scientific Sputter Coater 108) for 30 seconds under argon gas. Images were taken using 10-12 kV and saved as .TIF.

Chemistry Methods

Sample Preparation and Extraction

Three individual male *H. trizona* were included in the analysis. More replicates were not possible due to rarity of specimens. Each male was prepared for chemical extraction

by removing wings, antennae, proboscis, and legs from the body. The body was then macerated using fine scissors to ensure the solvent could gain access to the internal tissues. Flocculent was collected from each subabdominal pouch and extracted separately from the rest of the body. Tissues were placed in 10mL tubes in 5mL of dichloromethane (DCM). An internal standard was prepared using the PA Monocrotaline. Crystalized Monocrotaline provided by Frank C. Schroeder and was suspended in 0.1% Formic Acid in Water (FA:H2O) and serially diluted to 10uM. Each sample was spiked with 400µL (~1.3014 µg) of the Monocrotaline internal standard. Additionally, we prepared a sample that included only solvent and Monocrotaline standard to control for possible contaminants in our internal standard. Tissues were extracted at room temperature (25° C) for 36 hours.

After extraction, the supernatant from each sample was removed and filtered using a 0.45 µm PTFE filter membrane at 4500 RPM for 10 minutes. The filtered fluid was then aliquoted equally into two vials. These vials were then placed in a SpinVac and their solvent was evaporated. One vial was resuspended in 200µL of DCM for GC-MS analysis. The second vial was resuspended in 200µL of 0.1% FA:H2O for HPLC-MS/MS. At this stage, each sample had approximately 0.6507µg of the Monocrotaline internal standard at a concentration of 10µM. Samples were stored at -20°C until chemical analyses were performed.

Gas Chromatography Mass Spectroscopy (GC-MS)

We followed the GC-MS method used in previous publication dealing with the detection of male Lepidopteran pheromones (Komae et al., 1983). We used an HP-5ms column (30 m x 0.255mm, 0.25µm) on an Agilent 7890A GC equipped with an Agilent 5975C Mass Selective Detector. The temperature profile was programmed from 80° to

230°C at 4°C/min. We ran the machine splitless in selective ion monitoring mode, selecting the mass of the PA-derived tiger moth pheromone hydroxydanaidal (HD) (mass: 151.165 amu).

We compared the response of our samples to that of a stock solution of HD (860uM, provided by T. Hartmann) and a blank solution containing only DCM. We found that our method was not adequate for accurately reporting the concentration of HD from our samples, and so report only presence/absence data. The presence of HD was assessed by integrating the intensity of the GC response over the retention time of HD exhibited by our stock solution (Range: 22.5-24.0 min; Peak RT: 22.87±0.04 min.). If the area under the intensity curve at the retention time of HD was more than 6-fold that of the DCM blank solution, we assessed this as "presence".

Liquid Chromatography Mass Spectroscopy (HPLC-MS/MS)

Chemical analyses were carried out on a Thermo LTQ Orbitrap XL using a previously published method for detecting PAs (Avula et al., 2015). We used a Hypersil GOLD C18 Selectivity reversed-phase column (Thermo #25002-052130). Each sample and our internal standard control was run 6-7 times, with 0.1% FA:H2O blanks between each run to ensure cross contamination between runs did not occur. We loaded 20µL of each sample per run.

HPLC-MS/MS Data Analysis

We converted the proprietary Thermo .RAW files to the .MZXML format using msConvert (Chambers et al., 2012). These files were imported into MATLAB and analyzed using a custom script.

Putative PA Search Based on Fragmentation

A custom MATLAB script was written to perform the following analysis. All parent ions (MS1) between 155-440 amu that were detected within the first 24 minutes were analyzed. MS1's of the same mass (±0.099 amu) which eluted within 30 seconds of each other in a given run were considered to be the same MS1 and the most intense was selected to represent the group. For each MS1 the fragmentation spectrum (MS2) was reduced to include only the range of fragments between 93-175 amu, where most PAs contain shared fragment ions derived from their necine bases. We then recalculated the intensity of each fragment relative to the highest intensity fragment ion in this reduced range.

We compared the MS2 of each compound against a set of 8 clusters of fragments that are common to both free base and N-oxide forms of PAs (**Supplementary Table 1**). Clusters were designed such that typical PAs would only exhibit a particular subset of them and not others. This allowed for an additional level of filtering, as it was the specific combinations of clusters which identified PAs, not only the total number of fragment ions matched. A match was made when the MS1 of a putative PA shared at least one fragment ion of the same mass (±0.099 amu) with a given cluster.

After all clusters were compared a binary score or count was given for five characteristics: 1) presence of a PA-like cluster combination, 2) the total number of clusters matched, 3) the number of clusters matching a fragment ion with a relative intensity in the top 10%, 4) the number of clusters matching a fragment ion with a relative intensity in the top 10, and 5) whether the top 2 most intense fragment ions matched a cluster.

We then retained only MS1's as putative PAs when their MS2's met all of the following criteria: 1) contained a PA-like cluster combination, 2) ≥66% of all clusters were matched, 3) >80% of matched clusters contained a fragment ion with a relative intensity in the top 10%, 4) ≥50% of matched clusters contained a fragment ion with a relative intensity in the top 10, and 5) one cluster contained at least one of the top 2 most intense fragment ions. The only deviation was in the case of putative PAs that did not match our PA database (see below). In these cases, we set more stringent selection criteria, requiring all matched clusters to contained fragment ions with a relative intensity in the top 10%.

Compound Classification

No comprehensive PA library was available to us to cross-reference with our results. Therefore, we compiled our own database of >600 known PAs from a variety of bibliographic sources, mainly from the Dictionary of Alkaloids (Second Edition) (Buckingham et al., 2010). This database includes names and other chemical identifiers, chemical formulas, accurate mass information, and information about biological sources where known. To classify each putative PA we calculated the ppm difference between our measured masses and those in our database for known PAs. We retained classifications when the difference was less than 2 ppm.

When no match could be made, compounds were named as "Unknowns" (*e.g.*, Unknown 1). When an unmatched compound was found to have an N-oxide partner, we labeled both as "Compound" and either "Free Base" or "NOX" (*e.g.*, Compound 1 - Free Base). Because PAs often have multiple stereoisomers we cannot know their exact identity with certainty without employing other techniques. Due to this limitation, all stereoisomers

or PAs of identical mass to our named compounds should be considered as potential alternative classifications.

Two compounds were found to have very high ppm difference from their classified compounds (*i.e.*, "Platynecine N-oxide", "Retronecine"). We chose to classify these because their fragmentation spectra were manually verified to be PA-like and because of their relatively small mass few other PA compounds seem probable. We cannot currently explain why their masses deviated from their expected values by approximately 0.03 amu. However, we chose to place their names in quotations to denote lower confidence in their identifications and we separated their calculations from the compounds of similar mass, but lower ppm difference (*i.e.*, Platynecine N-oxide, Retronecine).

Filtering Putative PAs

After putative PAs were identified based on fragmentation spectra we filtered our results based on a number of criteria. First, we retained only compounds that were present in >80% of runs performed on each sample, allowing for 1 absence at most. We then retained only compounds that were detected at a 2-fold higher average concentration compared to both the solvent-only blanks and Monocrotaline-only standard. Finally, response ratio measurements from compounds with multiple retention times were summed.

Acoustic Recordings

Freshly captured moths were placed in a freezer on an ice block for no longer than 2-3 minutes until anesthetized. The wings were then folded above the thorax and secured using a hemostat. The moth was then allowed to return to ambient temperature and regain normal activity levels (*e.g.*, leg and antennal movement). All recordings were made in a

darkened room. Moths were stimulated to produce sound by playing a pre-recorded echolocation attack sequence from the sympatric insectivorous bat, *E. fuscus* (Arguero and Albuja, 2012). Other technical details of recording methodology and stimuli follow those previously published (Barber and Conner, 2006; Dowdy and Conner, 2016). Stimuli were repeated seven times per individual with approximately 4-5 seconds of silence between trials.

Results

Flocculent Structure

The flocculent structure is similar to that reported for *H. stictosoma* as well as *Gymnelia salvani, Sarosa sp., Pseudosphex polistes, Myrmecopsis crabronis,* and *Cosmosoma myrodora* (Boada, 1997; Conner et al., 2000; Yack et al., 2004). The flocculent of *H. trizona* is composed of flattened or slightly concave strands approximately 1.00-1.25mm in length and 10µm in width. The surface resembles an intricate network of reticulations (**Fig. 1B**). The only significant difference between the flocculent structure of *H. trizona* and that of other known flocculent-bearing species is that 5-7 individual fibers of flocculent appear fused into bundles (**Fig 1C inset**). The flocculent fibers are linked near their bases, close to where they are attached to the surface of the subabdominal pouch prior to release.

Moth Response to Restraint

We observed *H. trizona* to readily release flocculent in response to tactile stimulation (**Fig 1A; Supp. Video 1, 2**). The flocculent is everted by abdominal movements alone,

rather than by being scooped out by the legs as reported in *H. stictosoma*. We found that males were capable of approximately 3-4 flocculent releases in response to repeated restraint before both subabdominal pouches were emptied, though each subsequent release yielded less flocculent than the previous. As reported for *H. stictosoma*, the flocculent was found to be very sticky, adhering to most objects including metal, plastics, and human skin. We found that the flocculent of *H. trizona* was also very lightweight and would float on air currents in the room. Over the course of a few minutes, the individual bundles of flocculent would become unlinked from the large mass of flocculent and float around the room.

Moth Responses to Natural Bat Attacks

We were able to observe only a single interaction between an *H. trizona* male and a free-flying, captive bat. The bat captured and almost immediately dropped the moth, which landed safely on the leg of a tripod nearby. Trailing behind the moth was a mass of flocculent, confirming that flocculent is released when captured by bats, prompting the moth to be dropped unharmed (**Fig. 2**).

Chemical Analysis

GC-MS

We found that all samples contained some amount of HD, except the flocculent of individual 3 (**Table 1**). We were not able to quantify the HD from each sample in these experiments.

HPLC-MS/MS

We discovered the presence of 40 unique putative PAs in the body and flocculent of the three males examined. When free base and N-oxides pairs are combined, this represents 32 unique compounds (**Table 2**). The concentration of compounds varied markedly between samples (**Table 3**).

Surprisingly, the body tissues had relatively few compounds present. The bodies of individuals 1 and 2 contained only 10 and 9 putative PA compounds, respectively (**Table 4A**). The third body was completely devoid of any putative PA compounds. The chemical profiles were similar between samples, with body of individual 1 sharing 70% of its compounds with the body of individual 2.

Because not all compounds could be matched to known PAs, it is difficult to assess the proportion of non-toxic N-oxide forms to the toxic free base forms present in these tissues. An additional complication is that over time, free base forms can become reduced to their N-oxide state and thus any reported free base values would be a conservative estimate. However, of the compounds that were matched, both bodies containing PAs had 4 compounds in the free base form and 3 compounds in the N-oxide form. So at least 40-45% of the compounds found in the body tissues came in the form of the toxic free base.

The flocculent samples from each male contained a much larger suite of putative PAs compared to their respective body tissues. The concentrations of the putative PAs were also much higher in the flocculent samples than in the body tissues. This finding is even more stark when the mass of the flocculent relative to the body (~1-2%) is considered. Flocculent from individuals 1, 2, and 3 contained 20, 36, and 34 putative PA compounds, respectively (**Table 4B**). The chemical profiles were similar between samples, with the flocculent of individual 1 sharing 100% of its compounds with one or both of the other

flocculent samples, while the flocculent from the other individuals shared 94% of their compounds with one or both other individuals.

There were more toxic free base compounds recovered in the flocculent compared with the body tissues. Flocculent from individuals 1, 2, and 3 contained 8, 11, and 10 free base compounds, respectively. Proportionally this is a low number, however many of the putative compounds in the flocculent samples could not be matched to a known PA, and so this is likely an underestimate of the true number of free base PAs present. Free base forms made up approximately 50-60% of the putative PAs in flocculent that could be matched to the database.

Body and flocculent samples from the same individual shared some putative PAs, but flocculent samples contained a large number of unique compounds not recovered in their respective body tissues (**Table 5**). The flocculent of individuals 1 and 2 shared only 6 and 7 compounds with their respective body tissues. The only compounds shared between flocculent and bodies in both individuals 1 and 2 were Lycopsamine and Leptanthine N-oxide.

Overall, the most prevalent compound was Lycopsamine, or a steroisomer of it. This was the only compound recovered in all flocculent samples as well as 2 of the 3 body samples. Another notable constituent found in the flocculent was Callimorphine N-oxide, a PA known to be synthesized in the tissues of a number of tiger moths (Edgar et al., 1980). The free base and N-oxide forms of both Retronecine and Platynecine were also found in large amounts within most of the flocculent samples. These compounds often form the basis for more complex PAs of larger mass and are common in many PA-bearing plants.

Finally, Unknown 12 was also notable as having the highest concentration of all recovered putative PAs, though it was only found in the flocculent of individuals 2 and 3.

Acoustic Analysis

Moths were found to click in response to both simulated bat echolocation and tactile response as flocculent was being everted. Clicks were produced at a low rate compared with other tiger moth species (40 clicks/sec). The dominant frequency of the clicks was approximately 20kHz, however two additional peaks were present at 37kHz and 47kHz, within the range of those frequencies used in the echolocation of many bats.

Discussion

These results document the first case of flocculent being used against bat predators. The release of flocculent in this context appears to be capable of protecting this species from predation by bats.

The presentation of ultrasonic clicks both before and during the release of flocculent suggests moths may be using acoustic aposematism to warn bats of this defense, as other species of tiger moth have been demonstrated to do with other potent chemical defenses (Barber et al., 2009; Dowdy and Conner, 2016).

The putative PAs detected in the flocculent and the bodies of these moths, along with the rejection of *H. trizona* by a bat predator, suggest these moths are strongly chemically defended against predation. The most common PA among all individuals and tissue types was the toxic, free base form of Lycopsamine. This PA was also a major chemical component of *C. myrodora* along with its stereoisomer Intermedine (Conner et al., 2000). However, unlike *C. myrodora*, a large number of putative PAs were recovered from the

flocculent. The chemical profiles between individuals were similar, but not identical. The measured concentrations of most compounds varied greatly between individuals. The reason for this is not clear, however it is possible that *H. trizona* obtains PAs through adult pharmacophagy, a strategy employed by many tiger moths, including some Euchromiines (Zaspel et al., 2014). It may be that an individual's PA composition is dependent upon the PA sources they happen upon in their environment, generating some intraspecific variation in the realized chemical profile and concentration of its components. Invariably, individual males expressed greater PA diversity and chemical concentration in the flocculent as compared to their body tissues. The flocculent of *C. myrodora* was also noted to contain a much greater concentration of PAs relative to the body (Conner et al., 2000). Selective localization and concentration of defensive compounds has been observed in other Lepidoptera and is thought to maximize their deterrent effects on predators (Brower and Glazier, 1975).

It is not clear whether the defensive role of flocculent against bats requires the presence of PAs. The flocculent could act solely as a method for delivering their chemical defense to their bat predators. It is also possible that the flocculent acts as a physical defense, acting as a final escape maneuver. Likely, the chemical defense and the physical stickiness of the flocculent combine to disorient and disgust the would-be predator, allowing the moth to quickly escape. However, experiments comparing the proportion of dropped moths between reared individuals with and without access to PA sources would help clarify exactly what makes the flocculent a potent anti-bat defense.

Because we were only able to collect a small number of male *H. trizona*, we ablated the ears of the moths to increase our chances of observing a bat capture. If their ears were

intact it is possible that the moths could release flocculent in response to bat echolocation alone, before contact with a bat predator. The flocculent is very light and readily floats in the air. The flocculent bundles could function as biological analogue to chaff countermeasures used in human aerial warfare, distracting the bat and allowing the moth to escape. While we never observed *H. trizona* to release flocculent in response to simulated bat cries in the laboratory, those conditions do not simulate natural flight conditions. We believe this hypothesis should be tested in future experiments.

The flocculent is a limited resource. Once released, flocculent is not regenerated (Boada, 1997). It is not yet known whether this species utilizes flocculent in courtship as it is in species such as *C. myrodora*, *Syntomeida ipomoeae*, and *S. melanthus* (Sanderford, 1992; Conner et al., 2000; Johnson, 2002). However, we detected the PA-derived, male tiger moth pheromone, HD, in both the body and flocculent tissues of most individuals. This suggests that flocculent is used in the courtship of *H. trizona*, as it is in other Euchromiines, though this should be verified through direct observation of mating behavior in this species. If the flocculent is used in courtship, these moths would face an interesting trade-off. Male *C. myrodora* were significantly more likely to secure a mating from a female if they released flocculent during courtship (Conner et al., 2000). However, in order to find a female to court, males must first contend with bat predation. If too much flocculent is used in encounters with bats there may not be enough held in reserve to secure a copulation. However, if the flocculent is not utilized during a bat encounter, the male moth may not survive long enough to find a female to court. This system could provide unique insights into how a limited resource used for both survival and reproduction is managed.

Clearly, there is still much to be learned about the distribution of flocculent among tiger moths, its role in sexual courtship and defense, and its evolutionary origins.

Acknowledgments

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Figure 1. Flocculent emission and fine structure. A: Flocculent just after release in response to restraint with hemostats. **B:** SEM of a single strand of flocculent, detailing the surface structure. **C:** Bundles of connected flocculent strands. All scale bars are 10µm.

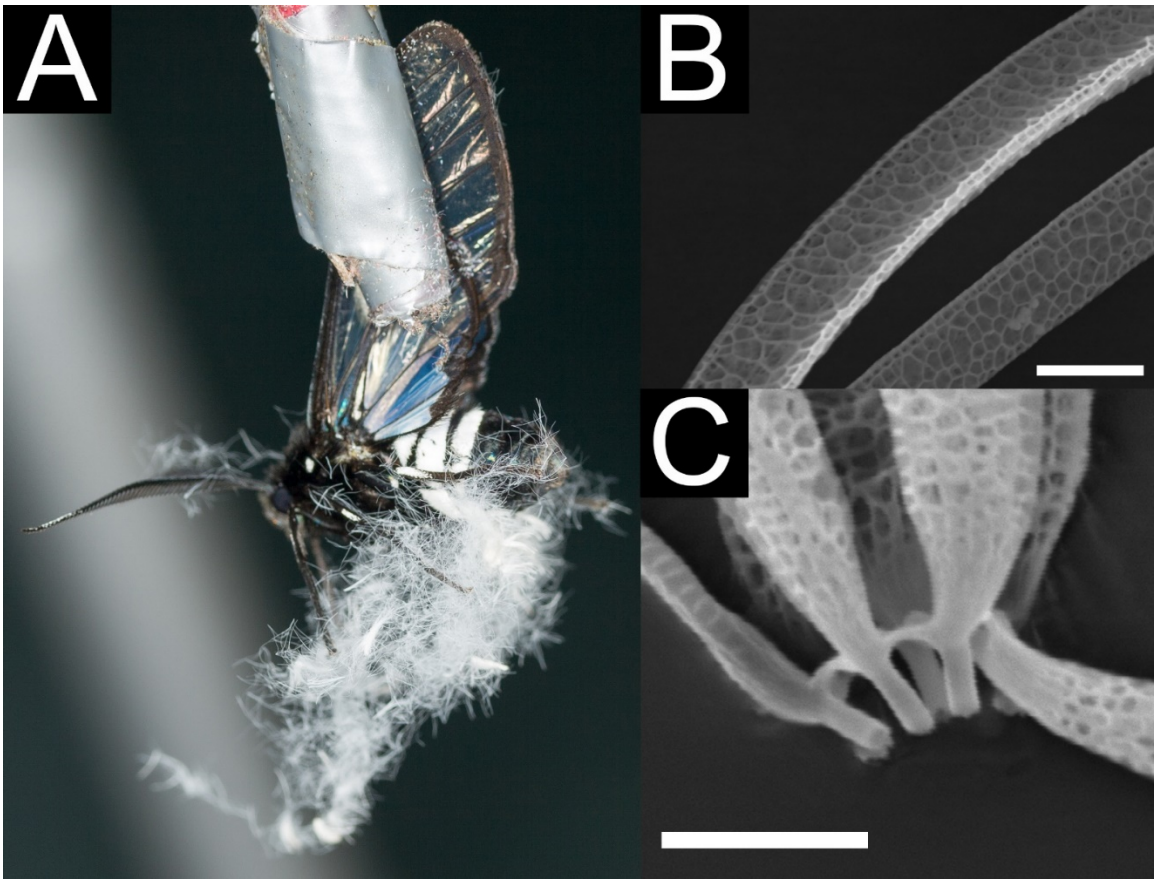

Figure 2. Flocculent release after bat capture. Arrow indicates the location of the flocculent. The image has had the brightness and contrast adjusted from the original.

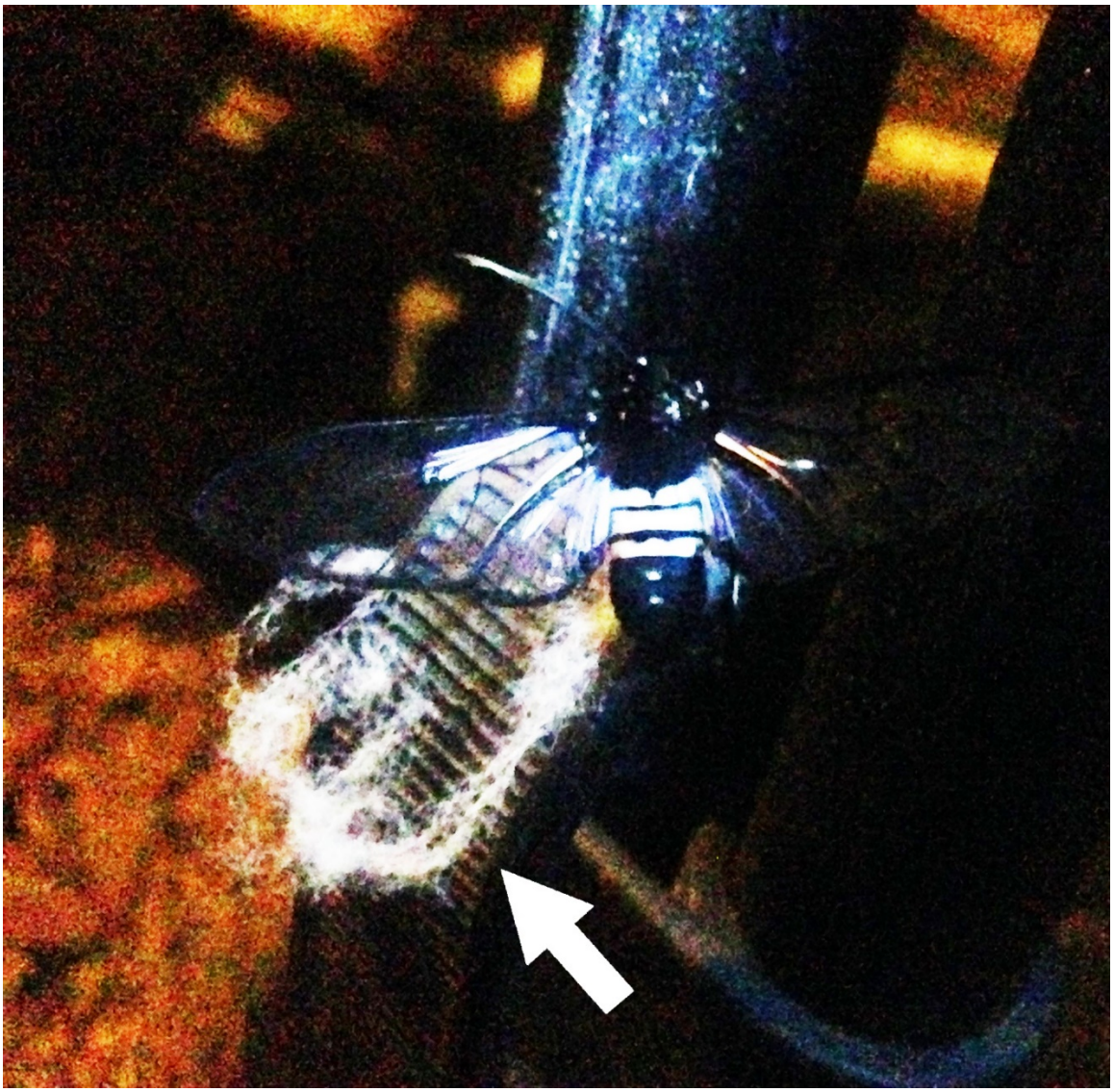

Table 1. GC-MS results (Continued on Next Page). PEAK RT refers to the retention time of the most intense part of the HD peak, where HEIGHT is measured. AREA is the integration of intensity over the retention time window spanning START RT to END RT. All retention times are measured in minutes. See also: https://doi.org/10.6084/m9.figshare.4964228

SPECIES	INDIVIDUAL	TISSUE	PEAK RT	HEIGHT	AREA	START RT	END RT	HAS HD
HODAL 860uM	NA	NA	22.760	4619241.2	226191.5	22.5	24	YES
DCM BLANK	NA	NA	23.145	869.3	151.2	22.5	24	NO
H. trizona	1	FLOCCULENT	22.881	65974.5	3779.3	22.5	24	YES
H. trizona	1	BODY	22.882	49682.5	2653.4	22.5	24	YES
H. trizona	2	FLOCCULENT	22.881	73506.1	8418.3	22.5	24	YES
H. trizona	2	BODY	22.871	6099.0	999.6	22.5	24	YES
H. trizona	3	FLOCCULENT	22.871	1634.9	195.3	22.5	24	NO
H. trizona	3	BODY	22.891	119325.9	14049.7	22.5	24	YES

Table 2. Putative PA compounds identified in three *H. trizona* males (Continued on Next Page). Dictionary masses come from Buckingham et al., 2010. Masses that exhibited multiple retention times were combined, but their retention times are reported. See Methods regarding “Platynecine NOX” and “Retronecine”. Mass measurements are given in amu. Retention time is given in minutes. See also: https://doi.org/10.6084/m9.figshare.4964228

Putative Compound	M+1 (Experimental)	Actual Mass (Experimental)	Actual Mass (Dictionary)	delta PPM	Retention Time (min)
Monocrotaline (Internal Standard)	326.1602	325.1529	325.1525	1.23	2.45
"Platynecine NOX"	174.1490	173.1417	173.1052	210.85	3.93
"Retronecine"	156.0770	155.0697	155.0946	160.55	0.47
Amabiline	284.1861	283.1788	283.1784	1.41	14.18
Callimorphine NOX	314.1602	313.1529	313.1525	1.07	14.02; 22.8
Compound 2 - Free Base	336.2385	335.2312	NF	NA	7.52; 7.92; 13.9
Compound 2 - NOX	352.2336	351.2263	NF	NA	8.78; 14.38; 15.1; 16.17; 17.37; 17.88
Compound 3 - Free Base	346.2230	345.2157	NF	NA	20.97; 22.47
Compound 3 - NOX	362.2180	361.2107	NF	NA	22.73
Compound 4 - Free Base	348.2386	347.2313	NF	NA	14.08; 19.47; 20.6; 22.62
Compound 4 - NOX	364.2336	363.2263	NF	NA	11.35; 17.02
Cropodine NOX	344.2064	343.1991	343.1631	1.07	11.86; 12.67
Curassavine	300.2173	299.2100	299.2097	1.00	10.85
Curassavine NOX	316.2099	315.2051	315.2046	1.38	9.3; 22.93
Heliocurassavicine	286.2015	285.1942	285.1940	0.70	19.87
Heliocurassavicine NOX	302.1961	301.1888	301.1889	0.25	10.37
Leptanthine	318.1916	317.1843	317.1838	1.58	8.97; 14.93
Leptanthine NOX	334.1867	333.1794	333.1788	1.80	22.87
Lycopsamine	300.1811	299.1738	299.1733	1.67	14.15; 23.33
Lycopsamine NOX	316.1759	315.1686	315.1682	1.27	15.73
Platynecine	158.1177	157.1104	157.1103	0.44	0.53; 1.73; 2.9; 4.27; 6.62
Platynecine NOX	174.1127	173.1054	173.1052	1.16	0.6
Retronecine NOX	172.0970	171.0897	171.0895	1.17	0.53
Strigosine	272.1861	271.1788	271.1784	1.29	10.1; 17.6
Strigosine NOX	288.1809	287.1736	NF	NA	14.65
Unknown 10	269.0548	268.0475	NF	NA	5.97; 7.35; 9.95; 11.18; 12.33
Unknown 11	274.1652	273.1579	273.1576	1.10	5.62; 6.7; 11.15
Unknown 12	283.1181	282.1108	NF	NA	23.67
Unknown 13	294.1914	293.1841	NF	NA	11.83; 13.45; 14.17
Unknown 14	318.2280	317.2207	NF	NA	17.57; 18.65; 20.45
Unknown 16	324.1424	323.1351	NF	NA	23.85
Unknown 17	328.2001	327.1928	NF	NA	18.13
Unknown 18	334.2229	333.2156	NF	NA	20.3; 20.92; 22.7; 22.85
Unknown 19	381.2239	380.2166	NF	NA	20.62; 21.33
Unknown 20	385.1649	384.1576	NF	NA	23.68
Unknown 21	392.2650	391.2577	NF	NA	19.02; 22.65; 23.22
Unknown 3	216.1597	215.1524	NF	NA	18.8
Unknown 4	220.1468	219.1395	NF	NA	2.1
Unknown 5	227.1281	226.1208	NF	NA	20.65
Unknown 7	255.1231	254.1158	NF	NA	18.52
Unknown 9	267.1231	266.1158	NF	NA	23.87

Table 3. Concentrations (µM) and standard deviations for each compound (Continued on Next Page). Concentration values are given as µM with their standard deviations. Flocculent from individual 2 was run 6 times. All other moth samples were run 7 times. Monocrotaline standard (MONO) was run 9 times. Blanks were run 23 times. Our internal standard, monocrotaline, is omitted from this table. See also: https://doi.org/10.6084/m9.figshare.4964228

			INDIVIDUAL 1		INDIVIDUAL 2		INDIVIDUAL 3	
Compound Area / ISTD Area (uM)	BLANK	MONO	BODY	FLOCCULENT	BODY	FLOCCULENT	BODY	FLOCCULENT
"Platynecine NOX"	0 ± 0	0 ± 0	0 ± 0	72.7 ± 12.1	0 ± 0	367.6 ± 81.7	0 ± 0	386.7 ± 294.9
"Retronecine"	0 ± 0	0 ± 0	18.4 ± 5.6	291.6 ± 86.9	0 ± 0	152.7 ± 62	0 ± 0	0 ± 0
Amabiline	0 ± 0	0 ± 0	0 ± 0	3.4 ± 4.5	0 ± 0	22.9 ± 23.4	0 ± 0	41.8 ± 48.1
Callimorphine NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	386.5 ± 205.1	0 ± 0	200.7 ± 210
Compound 2 - Free Base	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	60.9 ± 82.2	0 ± 0	509.6 ± 391.8
Compound 2 - NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	473.6 ± 198.6	0 ± 0	1717.3 ± 348.1
Compound 3 - Free Base	0 ± 0	0 ± 0	3.7 ± 2	8.2 ± 3	0 ± 0	19.5 ± 19.7	0 ± 0	275.7 ± 172
Compound 3 - NOX	0 ± 0	0 ± 0	7.8 ± 3.3	1.2 ± 1.7	4.2 ± 2	0 ± 0	0 ± 0	537.5 ± 538.7
Compound 4 - Free Base	0 ± 0	0 ± 0	13.9 ± 6.2	0 ± 0	8.4 ± 3.8	109.5 ± 150.9	0 ± 0	1249.5 ± 551.6
Compound 4 - NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	35.3 ± 35.3	0 ± 0	346.7 ± 115
Cropodine NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.7 ± 1.6	0 ± 0	0 ± 0
Curassavine	0 ± 0	0 ± 0	0 ± 0	5.2 ± 3.7	1 ± 0.8	28.9 ± 9.4	0 ± 0	29.4 ± 11.6
Curassavine NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	156.9 ± 190.1	0 ± 0	402.9 ± 406
Heliocurassavicine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	268.3 ± 62.7	0 ± 0	651.1 ± 419
Heliocurassavicine NOX	0 ± 0	0 ± 0	0.5 ± 0.3	0 ± 0	0.3 ± 0.2	12 ± 9.2	0 ± 0	37 ± 12.4
Leptanthine	0 ± 0	0 ± 0	0 ± 0	14.2 ± 2	0 ± 0	63 ± 17.3	0 ± 0	123.1 ± 40.8
Leptanthine NOX	0 ± 0	0 ± 0	1.3 ± 1.3	0.4 ± 0.3	0.7 ± 1	3.2 ± 4.1	0 ± 0	0 ± 0
Lycopsamine	0 ± 0	0 ± 0	2.4 ± 0.6	5.4 ± 0.8	1.4 ± 0.4	293.3 ± 101.5	0 ± 0	508.9 ± 194.3
Lycopsamine NOX	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3.7 ± 3.2	0 ± 0	0 ± 0
Platynecine	0 ± 0	0 ± 0	0 ± 0	202.3 ± 27	10.3 ± 15.4	553 ± 171.8	0 ± 0	706.4 ± 338.9
Platynecine NOX	0 ± 0	0 ± 0	0 ± 0	117.4 ± 33.4	0 ± 0	201.4 ± 41.7	0 ± 0	72.2 ± 49.5
Retronecine NOX	0 ± 0	0 ± 0	0 ± 0	34.9 ± 8.8	0 ± 0	74.7 ± 18.1	0 ± 0	28.9 ± 12.8
Strigosine	0 ± 0	0 ± 0	0 ± 0	6.5 ± 3.7	0 ± 0	509.4 ± 397.9	0 ± 0	700.7 ± 496.8
Strigosine NOX	0 ± 0	0 ± 0	0 ± 0	199.6 ± 20.7	0 ± 0	911.9 ± 214.1	0 ± 0	563.7 ± 486.1
Unknown 10	0 ± 0	0 ± 0	0 ± 0	10.8 ± 7.3	0 ± 0	273.6 ± 171.8	0 ± 0	864.8 ± 417.1
Unknown 11	0 ± 0	0 ± 0	0 ± 0	8.7 ± 2.2	0 ± 0	207.8 ± 122.4	0 ± 0	26.9 ± 17.7
Unknown 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	957.7 ± 982.3	0 ± 0	3482.1 ± 2919.9
Unknown 13	0 ± 0	0 ± 0	0 ± 0	119.7 ± 57.4	0 ± 0	453.1 ± 379.4	0 ± 0	1809.1 ± 520.6
Unknown 14	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	430.6 ± 327.5	0 ± 0	1339.7 ± 519.6
Unknown 16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	186.4 ± 42.6	0 ± 0	61.2 ± 21.6
Unknown 17	0 ± 0	0 ± 0	5.7 ± 1.5	0 ± 0	2.8 ± 1	0 ± 0	0 ± 0	0 ± 0
Unknown 18	0 ± 0	0 ± 0	6.9 ± 2.8	7.7 ± 5.8	0 ± 0	296.9 ± 326.8	0 ± 0	1485.7 ± 999.1
Unknown 19	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	315.6 ± 74	0 ± 0	369.4 ± 192.1
Unknown 20	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	677.4 ± 390.1
Unknown 21	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	290.7 ± 279.3	0 ± 0	685.9 ± 394.5
Unknown 3	0 ± 0	0 ± 0	0 ± 0	13.7 ± 4.4	0 ± 0	65.3 ± 33.8	0 ± 0	80.6 ± 52.2
Unknown 4	0 ± 0	0 ± 0	16.4 ± 0.9	0 ± 0	10.2 ± 0.8	36.6 ± 14.2	0 ± 0	250.3 ± 69.2
Unknown 5	0 ± 0	0 ± 0	0 ± 0	9.5 ± 4	0 ± 0	68.1 ± 15.4	0 ± 0	173 ± 76.7
Unknown 7	0 ± 0	0 ± 0	0 ± 0	11.1 ± 1.2	0 ± 0	46.8 ± 9.6	0 ± 0	157.6 ± 101.3
Unknown 9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	572 ± 453.8

Table 4. Profile comparisons of tissue types between individuals. "B" refers to "Body" and "F" refers to "Flocculent". Numbers correspond to the individual. UNIQUE denotes the number of compounds unique to a given tissue/individual. Intersection symbols denote shared compounds between indicted tissue/individual. PA COUNT are the total number of compounds in each tissue/individual. See also: https://doi.org/10.6084/m9.figshare.4964228

TABLE 4A	UNIQUE	B1 ∩ B2	B1 ∩ B3	B2 ∩ B3	B1 ∩ B2 ∩ B3	SUM	PA COUNT
B1	30%	70%	0%	0%	0%	100%	10
B2	22%	78%	0%	0%	0%	100%	9
B3	0%	0%	0%	0%	0%	0%	0

TABLE 4B	UNIQUE	F1 ∩ F2	F1 ∩ F3	F2 ∩ F3	F1 ∩ F2 ∩ F3	SUM	PA COUNT
F1	0%	10%	0%	0%	90%	100%	20
F2	6%	6%	0%	39%	50%	100%	36
F3	6%	0%	0%	41%	53%	100%	34

Table 5. Profile comparisons of tissue types within individuals. Conventions follow Table 4. See also: https://doi.org/10.6084/m9.figshare.4964228

TABLE 5A	UNIQUE	B1 ∩ F1	SUM	PA COUNT
B1	40%	60%	100%	10
F1	71%	29%	100%	21

TABLE 5B	UNIQUE	B2 ∩ F2	SUM	PA COUNT
B2	22%	78%	100%	9
F2	81%	19%	100%	36

TABLE 5C	UNIQUE	B3 ∩ F3	SUM	PA COUNT
B3	0%	0%	0%	0
F3	100%	0%	100%	35

Supplementary Table 1. Clusters and their constituent fragment ions used in searching for PA-like compounds. Cluster fingerprints used were (1, 3, 6), (1, 5, 6), (1, 3, 5, 6), (2, 3, 5, 6), (4, 7, 8). All values are given in amu. See also: https://doi.org/10.6084/m9.figshare.4964228

	FRAGMENT 1	FRAGMENT 2	FRAGMENT 3	FRAGMENT 4	FRAGMENT 5
CLUSTER 1	92.9225	93.9111	94.9415	95.9456	96.9697
CLUSTER 2	104.9627	105.9909	107.0010	-	-
CLUSTER 3	107.9610	108.9488	109.9400	-	-
CLUSTER 4	111.9641	112.9739	113.9900	114.9300	-
CLUSTER 5	117.9846	118.9960	119.9479	120.9892	-
CLUSTER 6	122.0015	123.0150	124.0758	126.0000	-
CLUSTER 7	136.9839	137.9740	139.0510	140.0420	141.0245
CLUSTER 8	153.9499	155.0130	156.0270	157.0760	158.0760

CHAPTER V

A NOVEL ANTI-BAT FUNCTION OF PHEROMONE DISSEMINATING STRUCTURES IN THE TIGER MOTH EUCEREON ZIZANA (LEPIDOPTERA: EREBIDAE: ARCTIINAE)

Research Article

A novel anti-bat function of pheromone disseminating structures in the tiger moth *Eucereon zizana* (Lepidoptera: Erebidae: Arctiinae)

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Abstract

Many male tiger moths (Erebidae: Arctiinae) possess paired, eversible tube-like abdominal organs called coremata. Typically, these structures play a role in sexual communication by disseminating pheromones such as the pyrrolizidine alkaloid (PA) derivative hydroxydanaidal (HD) during courtship. These pheromones can be important indicators of male quality as they are derived from sequestered plant toxins (PAs) that contribute to anti-predator defenses by rendering the moths, their mates, and their offspring unpalatable to predators. We here describe a novel behavior where males of *Eucereon zizana* evert their coremata in response to simulated bat predation. We found that these moths everted their coremata in response to tactile stimulation and bat echolocation significantly more often than white noise or the sounds of conspecifics. We also performed palatability tests with free-flying bats and chemical analyses to determine whether these moths are chemically defended. We found that male *E. zizana* were highly unpalatable, they sequester a suite of PAs, and their coremata contain HD. This is the first evidence of a Lepidopteran co-opting a pheromone disseminating structure typically involved in sexual courtship for use against a predator. This anti-bat corematal display of HD may function as an aposematic odor, honestly signaling the moth toxicity.

Keywords: *Eucereon zizana*, Coremata, Arctiinae, Anti-predator defense, Hydroxydanaidal

Introduction

Tiger moths (Erebidae: Arctiinae) are a unique group of animals that serve as model organisms for understanding not only natural selection, but also sexual selection. This diverse moth lineage is best known for sequestering host plant toxins for use in both sexual communication and as anti-predator defenses, often signaled by aposematic colors and/or sounds. To aid in sexual communication, males of many species have evolved specialized structures called coremata.

Coremata are paired, eversible tube-like organs that are located on the ventral surface of the male abdomen between the 7th and 8th sterna. The most recent ancestral state reconstruction suggests coremata evolved once within the Arctiinae and were subsequently lost in some lineages (Simmons et al., 2012). Their main function is to disseminate male sex pheromones such as hydroxydanaidal (HD) to aid in mate finding, forming aggregations, and in some cases female mate choice (Birch et al., 1990). In all tiger moth species examined to date, the production of HD is contingent on the dietary acquisition of pyrrolizidine alkaloids (PAs) (Davidson et al., 1997). These alkaloids render adults, eggs, and larvae unpalatable thereby protecting them from predation by both vertebrate and invertebrate predators or parasitoids (Dussourd et al., 1988; Eisner & Eisner, 1991; Hare & Eisner, 1993; Eisner et al., 2000; Bezzerides et al., 2004; Dowdy & Conner, 2016).

We offer a new view of the defensive use of coremata and HD in tiger moths. We describe the corematal eversion behavior of the neotropical species, *Eucereon zizana*, in response to bat attack echolocation and tactile stimulation. This is the first evidence of a

tiger moth utilizing corematal structures and HD in a context unrelated to sexual communication.

Methods

Ethics Statement

Some vertebrates (bats) were captured or handled during these experiments. The methods employed in this study were approved by the Ecuadorian government (Permit 003-15-IC-FAU-DNB/MA issued by the Ecuadorian Ministry of Environment). The methods of this study were approved by the Wake Forest University Institutional Animal Care and Use Committee (protocol #A14-091). This work was performed with permission on private property.

Field Site and Insect Capture Method

Field experiments were conducted at the Yanayacu Biological Station and Center for Creative Studies (YBS) approximately 5 km west of Cosanga, Ecuador. The GPS coordinates of the field site are: 00°36.235' S, 77°52.917' W; elevation: 2,100 m. This location was chosen based on the impressive amount of information available regarding the life history and identities of its high diversity moths (Rab-Green et al., 2011). YBS lies on the eastern slopes of the Andes and is comprised of primary forest as well as partially reforested pastures and roadsides.

Insects were collected on station grounds from sheets illuminated with 15 Watt ultraviolet “quantum” lights (Leptraps.com; F15T8QBL). *Eucereon zizana* (Dognin, 1897) males were collected April 8th-14th, 2015 and placed individually in 30mL plastic containers and stored for up to 24 hours at ambient temperatures (10-16° C). Both male

and female *E. zizana* are commonly collected in light traps at this field site, however much of the natural history of this species is unknown. Information about host plants and possible pharmacophagous sources is scarce. A single female from this location has been reared from *Chusquea scandens* (Poaceae) (Rab-Green, pers. comm.). This species has noted to occur in Colombia, Ecuador, Peru, and Bolivia. *E. zizana* males have a mean forewing length of 1.55±0.01 cm (n=23). These moths are cryptically colored, with predominantly grey and white fore- and hindwings. (**Figure 1A**). The coremata in this species are single-branched, covered in elongate androconial scales, and are approximately 1 cm in length when fully everted (**Figure 1B**).

Acoustic Recordings

Freshly captured moths were held by the wings folded above the thorax and using a hemostat. All recordings were made in a darkened room. An Avisoft Bioacoustics USGH digital recording unit was connected to a single Avisoft CM16/CMPA ultrasonic microphone (± 3 dB from 15-140 kHz) and set to record at a sampling rate of 250 kHz. The microphone was placed perpendicular to the midline of the moth body, 10 cm from the thorax of the individual (where the sound-producing organs are located). An AT100 ultrasonic speaker (Binary Acoustic Technology) was placed 10 cm from the posterior end of the moth thorax (where the tympanal hearing organs are located), parallel to the midline of the body. Moths were stimulated to produce sound by playing a pre-recorded echolocation attack sequence from the sympatric insectivorous bat, *E. fuscus* (Arguero and Albuja, 2012). We characterized the anti-bat acoustic response by averaging 6 responses of 3 male *E. zizana*. For maximum duty cycle calculations we averaged 9 responses from 5 moths. For more details see previously reported methods (Barber & Conner, 2006).

Definitions of the acoustic parameters we measured follow those previously reported (Corcoran et al., 2010).

Moths were stimulated to evert their coremata with playbacks of four pre-recorded acoustic stimuli:

1. Echolocation attack sequence from the sympatric insectivorous bat, *E. fuscus*
2. White noise (0-125kHz range)
3. Conspecific female responses to tactile stimulation
4. Conspecific male responses to tactile stimulation

In addition, we used tactile stimulation to elicit corematal eversion by gently touching the head of the moths with a paintbrush.

For each individual (n=31) we generated a random sequence for stimulus presentation using the base functions of R (R Core Team, 2016). In total, twenty stimuli were presented to each moth with 5-10 seconds of rest between each stimulation. Tactile stimulation, white noise, and simulated bat attacks were each presented with approximately 25% frequency (approximately 5 presentations each). Male and female conspecific playbacks were each presented with about 12.5% frequency (approximately 2-3 presentations each). We refer to the eversion of the coremata in response to a stimulus as a positive response which was scored as a “1” for the purposes of statistical analyses. The lack of corematal eversion is a negative response and was scored as a “0”.

We conducted another test to ensure that the moths were not simply responding most strongly to the acoustic stimulus they were first presented with. We divided the moths into two groups which either: 1) received bat cries first, white noise second (n=17), or 2)

received white noise stimulus first, bat cries second (n=14). For each moth we sequentially presented the assigned stimulus five times with 5-10 seconds of rest between stimulations and recorded whether the corematal eversion response occurred. We then presented the second stimulus in the same manner and recorded the moths' responses.

Palatability

Bats were trained to capture free-flying, palatable "control" species of moths around a work light in a custom enclosure over the course of approximately one week. Initially, control moths were continuously added to the enclosure in large numbers such that the bats had a practically unlimited supply of moths to feed on. The number of free-flying moths was gradually reduced and replaced with control moths that were tossed into the air by hand around the work light. In the final stages of training, bats fed consistently on control moths tossed into the air while continuing to supplement their feeding with the remaining free-flying control moths in the enclosure.

Once trained to consistently take control moths tossed into the air, "experimental" *E. zizana* moths were added to the routine. We used the base functions of R to generate a random presentation order of control moths and experimental moths. Moths were presented to bats by tossing them up into the air approximately 1-2 meters in front of the bat. Control and experimental moths were presented after incapacitation via thoracic compression to remove any effects of corematal eversion on palatability. Interactions were scored on whether bats dropped the moths (score=0; "unpalatable") or if they ate the moths (score=1; "palatable").

Trials continued until bats showed signs of satiation which was defined as two sequential and complete rejections of control moths or until bats ceased flying. Trials began again after 1-2 hours or when the bat began flying again, whichever occurred first.

Statistics

Statistical analyses of the data were performed in R version 3.3.2 (R Core Team, 2016). Means are reported with their standard errors unless otherwise noted. An alpha level of 0.05 was used as a significance threshold for all statistical tests. To compare the frequency of corematal eversion between our five stimuli we utilized a generalized linear mixed effects model with a binomial error distribution and a logistic link function implemented in the lme4 package of R (Bates et al., 2015). Our fixed effect was stimulus type and our random effect was the ID of each moth. This random effect accounts for individual variation in corematal eversion frequency. Our model took the form: Corematal Eversion ~ Stimulus Type + (1 | Moth ID).

Palatability was assessed using the Exact Binomial Test. Acceptances by bats were coded as "successful" trials, the hypothesized null probability of acceptance was 100% (perfectly palatable), and the alternative hypothesis was that the true probability of acceptance was less than 100%.

Other Methods

Three male *E. zizana* were used in these chemical analyses. The tip of the abdomen, including the coremata was excised from the rest of the body of each male using iridectomy scissors and analyzed separately from the rest of the body. The body of Individual 1 could not be included in this analysis because the material was consumed in the process of

designing and testing the chemical methods used in this study. Chemistry results are reported as means with standard deviations. Detailed information about methodology related to bat capture and housing, chemistry, scanning electron microscopy follow those published elsewhere (see **Chapter 4, this text**).

Results

Corematal Eversion

Male *E. zizana* (n=31) everted their coremata in response to tactile stimulation in 86±2% (mean ± 95%CI) of trials (n=132/154), to bat cries in 58±4% of trials (n=94/162), to white noise in 20±3% of trials (n=29/147), to female conspecifics in 16±3% of trials (n=14/90), and to male conspecifics in 12±3% (n=8/67) (**Figure 2**). A generalized linear mixed effects model showed that tactile stimulation produced corematal eversion significantly more frequently than did bat cries and that bat cries were more significantly more effective at eliciting corematal eversion than white noise, female conspecifics, and male conspecifics. No significant differences were found between white noise and conspecific sounds (**Table 1**).

Acoustic Analysis

Male *E. zizana* activate their tymbal organs (**Fig 1C**) in response to the echolocation attack sequence of the big brown bat, *Eptesicus fuscus*. Sounds (**Fig 1D-G**) are typical of arctiine erebids in that they are composed of a series of broadband clicks produced during flexion of the tymbal (active modulation half-cycle) and during relaxation of the tymbal (passive modulation half-cycle). *E. zizana* sounds exhibit a dominant peak frequency of 34.4±0.64 kHz and a maximum duty-cycle of 8.2±0.9%.

This species has only about 4 microtymbals and produced only 2.1±0.2 clicks per half-modulation cycle. We did not have access to the equipment necessary to accurately determine the intensity of these clicks. Clicks occurred during the early approach through the buzz phase and overlapped with corematal eversion. We did not have access to high-speed video/audio synchronization equipment to precisely quantify when coremata were everted relative to the bat attack sequence. However, based on visual observations, we estimate that 86% (n=12/14) of corematal eversions occurred during or immediately after the buzz phase.

Chemical Analysis

GC-MS

We found that all samples contained some amount of HD, except the body of individual 2 (**Table 2**). We were not able to quantify the HD from each sample in these experiments.

HPLC-MS/MS

We discovered the presence of 41 unique putative PAs in the body and flocculent of the three males examined. When free base and N-oxides pairs are combined, this represents 32 unique compounds (**Table 3**). The concentration of compounds varied markedly between samples (**Table 4**).

The body tissues contained more putative PAs than most of the coremata samples. The bodies of individuals 2 and 3 contained 18 and 15 putative PA compounds,

respectively (**Table 5A**). The chemical profiles were similar between samples, with body of individual 2 sharing 61% of its compounds with the body of individual 3.

Because not all compounds could be matched to known PAs, it is difficult to assess the proportion of non-toxic N-oxide forms to the toxic free base forms present in these tissues. However, of the compounds that were matched, both bodies had 8-9 compounds in the free base form. Body 2 had 6 compounds in the N-oxide form, whereas Body 3 had only 3. So at least 45-60% of the compounds found in the body tissues came in the form of the toxic free base.

The coremata samples generally contained fewer putative PAs compared with their respective body tissues (**Table 5B**). However, the coremata from Individual 1 contained 27 putative PA compounds, a much larger number as compared to the other coremata and body samples. The chemical profiles were generally similar between samples, with the coremata of individual 2 sharing 100% of its compounds with one or both of the other coremata samples. The coremata of individual 3 was also similar to the other coremata, sharing 86% of compounds.

There were slightly fewer number of toxic free base compounds recovered in the coremata compared with the body tissues. Coremata from individuals 1, 2, and 3 contained 8, 6, and 3 free base compounds, respectively. However, as a proportion of the total number of compounds detected in each sample, the body and coremata have approximately the same ratio of free base to N-oxide forms. Toxic free base forms made up approximately 30-60% of the putative PAs in coremata that could be matched to the database.

The correspondence in putative PA profiles between tissue types within individuals varied (**Table 6**). The coremata of Individual 2 shared 80% of its profile with its body tissues, however the coremata of Individual 3 only shared 29% with its body tissues. The only compound shared between all coremata and body samples was the free base form of Compound 4. However, Compound 3 (both forms), Heliocurassavicine N-oxide, Lycopsamine, Retronecine, Leptanthine N-oxide, and Unknown 14 were prevalent in most samples. Where bodies and coremata shared compounds, the concentrations were generally on the same order of magnitude. The most concentrated compounds overall include Unknown 12, Platynecine (both forms), Strigosine N-oxide, Unknown 13, and Callimorphine N-oxide.

It is interesting that the free base and N-oxide forms of Platynecine were found in much larger concentrations as compared to those of Retronecine and may indicate something about the host plants utilized by this species.

Palatability Results

Male *E. zizana* were highly unpalatable. In tests with two individual male *Myotis sp.*, *E. zizana* were rejected in 100% (n=16) of trials (Exact binomial test: $p < 0.01$, 95% CI = [0.00, 0.17]). Control moths (n=49) were always accepted whenever they were presented to either bat (Exact binomial test: $p = 1$, 95% CI = [0.99, 1.00]).

Discussion

E. zizana males frequently evert their coremata in response to bat attack echolocation as well as when they are touched or physically restrained, as would occur when captured by a bat. In both the lab and the field, the major function of the acoustic

emissions of tiger moth species exhibiting low duty-cycle signals has been shown to be aposematic or mimetic signaling of unpalatability (Dunning, 1968; Acharya & Fenton, 1992; Dunning et al., 1992; Dunning & Kruger, 1995; Ratcliffe & Fullard, 2005; Hristov & Conner, 2005; Barber & Conner, 2007; Barber et al., 2009; Dowdy & Conner, 2016).

The simultaneous production of clicks and corematal eversion during the late stages of bat attacks along with the palatability and chemistry results indicate that the coremata may play a role in enhancing acoustic aposematic signaling or perhaps even acting as aposematic signals of their own. Research in other insects has shown that the defensive odors produced in response to disturbance could themselves act as warning signals (Rothschild, 1961). This phenomenon has been termed “odor aposematism”, “olfactory aposematism”, or “chemical aposematism” (Eisner and Grant, 1981; Weldon, 2013). Most research has focused on pyrazine as a common aposematic odor, however the PA-derived HD would also seem a natural candidate (Woolfson and Rothscild, 1990; Rothschild et al., 1984; Kaye et al., 1989; Vencl et al., 2016).

The neotropics contain the planet’s largest concentration of tiger moth diversity (~6,000 species), most of which likely produce acoustically aposematic or mimetic signals (Watson and Goodger, 1986). Because HD is only known to be derived directly from sequestered PA compounds, its presence is a strong indicator of the presence of PAs. Using HD pheromone emitted from the coremata as an honest signal of toxicity could be one method of avoiding Batesian or quasi-Batesian mimicry in an ecosystem filled with other sound-producing moth species.

Among insectivorous bats within the family Vespertilionidae olfaction is sometimes utilized for tasks such as individual, kin, and group recognition (Bloss, 1999).

A recent review of the sensory biology of bats concluded that, relative to other mammals, bats have not lost a significant proportion of olfactory receptor genes, the number of which is thought to correlate with a species' reliance on olfaction (Jones et al., 2013). It has even been suggested that the New Zealand short-tailed bat, *Mystacina tuberculata* may use olfactory cues while hunting insect prey when foraging terrestrially (Jones et al., 2003). However, a study of the sensory basis of prey detection among three species of palaeotropical insectivorous bats found that only acoustic cues were used in determining whether prey would be captured (Schmieder et al., 2012). Therefore, it seems that olfaction of prey from a distance by aerially hawking bats is unlikely. To our knowledge no studies have yet examined what role, if any, short-range, post-capture olfactory or gustatorial cues may play in determining whether prey will be consumed or rejected. These studies are needed to understand how bats determine the palatability of the prey they capture.

Acoustic signals play an important role in the sexual courtship of a number of tiger moth species (Conner, 1987; Conner, 1999; Sanderford & Conner, 1990; Sanderford & Conner, 1995; Sanderford et al., 1998; Simmons & Conner, 1996). It has been shown that females of some moth species are unable to distinguish conspecific male sounds from those of bat predators. Upon hearing the sounds of conspecific males, the females lie still, becoming easier for males to copulate with (Nakano et al., 2010; Nakano et al., 2013). Clicks of females produced during courtship might elicit male corematal eversion, and bat echolocation could resemble those sounds. However, in our experiments, males did not frequently evert their coremata when stimulated with female nor male conspecific sounds. Therefore, it is unlikely that the corematal eversion in response to bat

echolocation is a by-product of similarities between the sounds of bats and conspecifics. While the tested signals are not courtship-specific emissions, we expect them to be similar. In *Cycnia tenera*, a tiger moth known to use sound in courtship and as an anti-bat defense, these sounds differ only in the duration of production. Courtship sounds last approximately 4-8 seconds while anti-bat sounds occur for approximately 1-3 seconds during the approach phase, buzz phase, and sometimes while being handled during a bat attack (Conner, 1987; Dowdy, pers. obs.).

We observed that corematal eversion occurs in the late stages of the bat attack sequence and *E. zizana* males responded less to white noise compared to bat echolocation. We believe this indicates that a high duty-cycle acoustic signal that is also appropriately arranged temporally is necessary to cause corematal eversion. The duty-cycle threshold and temporal pattern required is currently unknown, but because both male and female *E. zizana* produce low-duty cycle clicks (~8%), we expect female conspecifics are unlikely to be able to produce the necessary signals.

Predation experiments exposing free-flying bats to *E. zizana* males with and without the ability to evert their coremata are necessary to determine how corematal eversion in response to bat echolocation affects predation risk. In addition, it will be necessary to compare the predation risk of males reared with and without access to PAs to address whether the odor component (*i.e.*, the PA-derived pheromone HD) is vital to the efficacy of this anti-bat behavior.

This is the first evidence of a lepidopteran co-opting a pheromone disseminating structure typically involved in sexual courtship for use against a predator. This unique behavior illustrates the diversity of anti-predator strategies employed by insects. More

broadly, it serves as a reminder of how much we still have yet to learn about the frequency of exaptations and their importance in the evolutionary origins of novel survival strategies.

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Figure 1. Morphology and acoustic emissions of *E. zizana* (Continued on Next Page). The moth (**A**) and tymbal organ (**B**), everted coremata (C), oscillogram (**D**), spectrogram (**E**), power spectral density plot (**F**), and the spectrogram of their response to simulated bat cries (**G**) are shown. Oscillogram, spectrogram, and power spectral density plots (**D-F**) show a single activation and relaxation (modulation cycle) of the tymbal organ. Moth responses to simulated bat cries (**G**) show each species' earliest response. Bat cries are brightest and sweep from higher to lower frequencies within a single call. Moth clicks are broadband and cluster in groups of clicks.

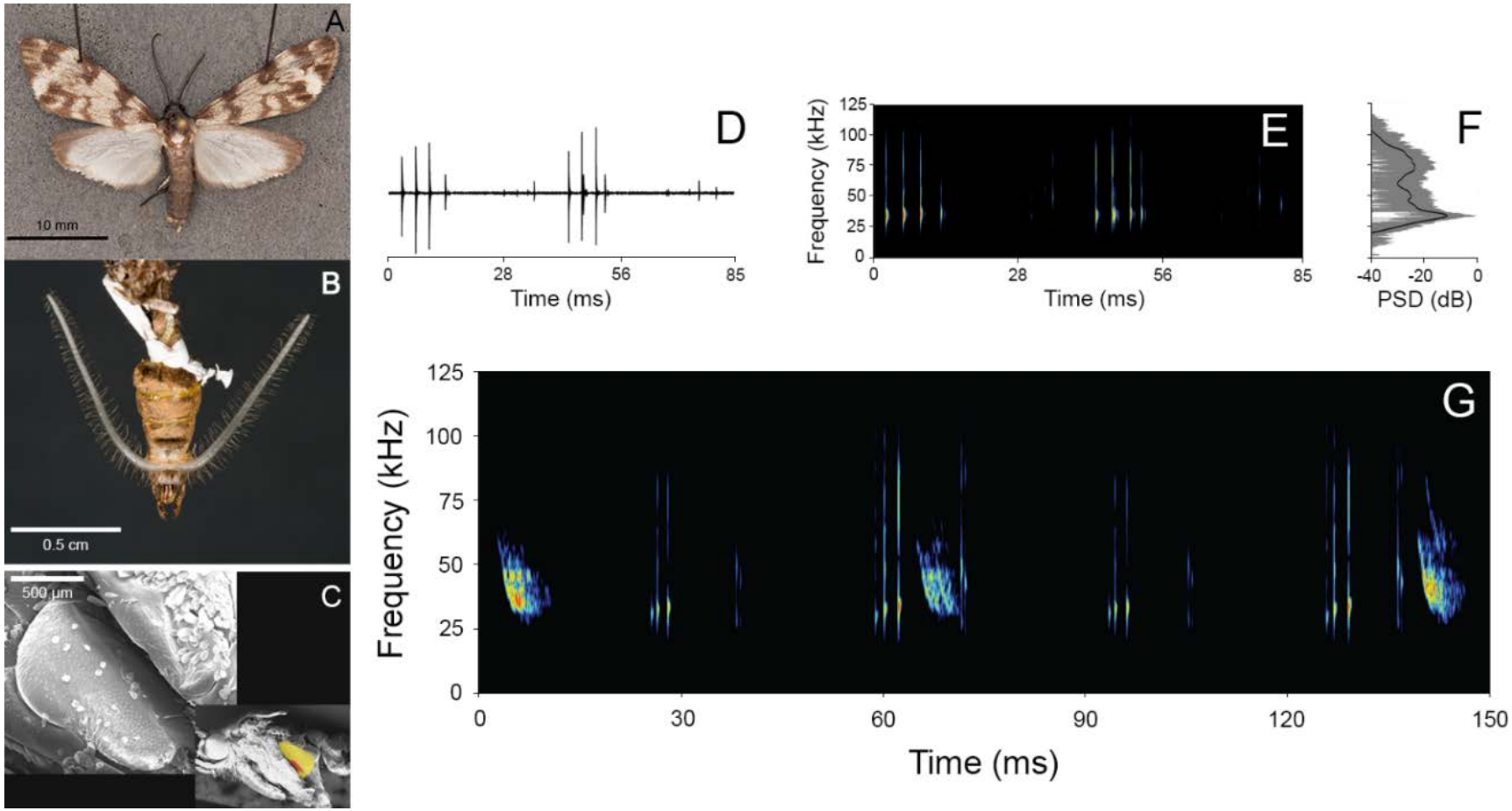
A
10 mm
B
0.5 cm
C
500 µm
D
0
28
56
85
Time (ms)
E
Frequency (kHz)
125
100
75
50
25
0
0
28
56
85
Time (ms)
F
-40
-20
0
PSD (dB)
G
Frequency (kHz)
125
100
75
50
25
0
0
30
60
90
120
150
Time (ms)

Figure 2. Frequency of corematal eversion by stimulus type. Letters signify significantly different groups. Points represent mean response frequency and bars represent 95% CI intervals on the mean.

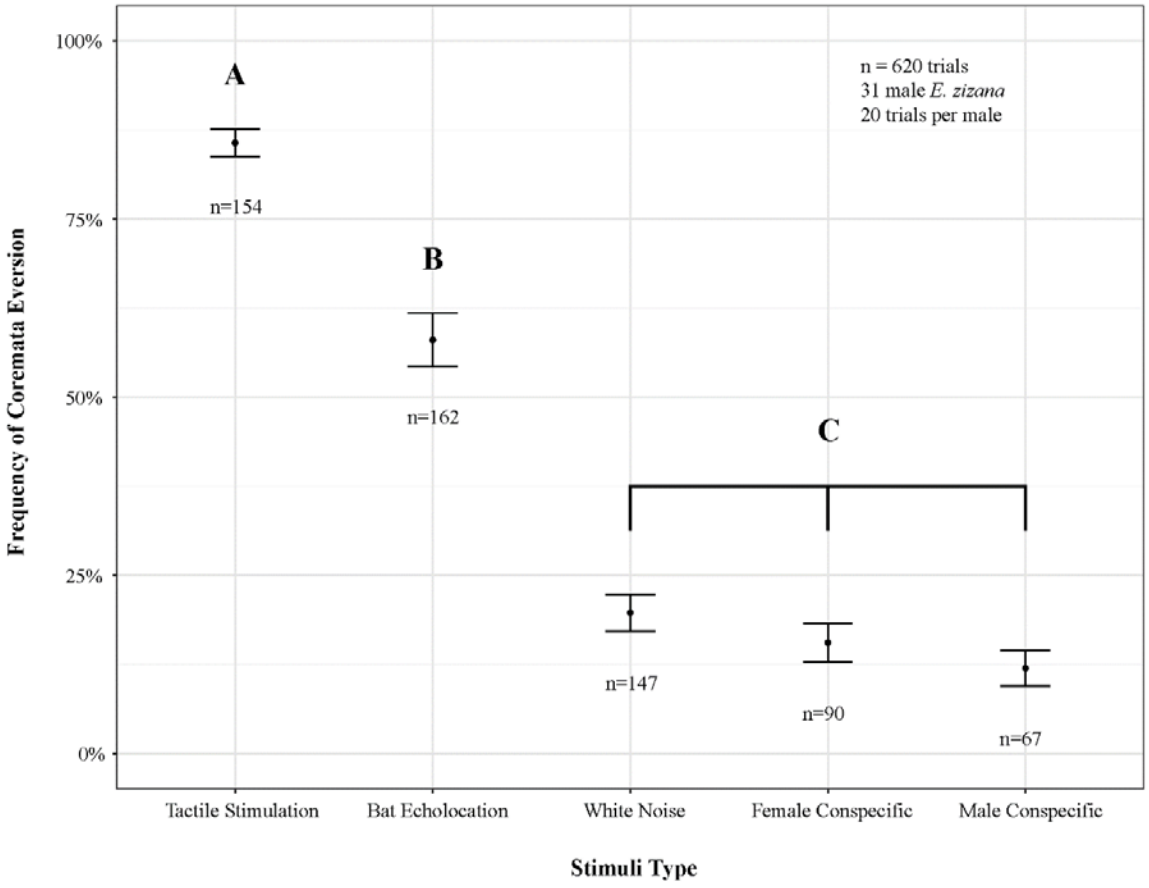

Table 1. Generalized Linear Mixed Effects Model Results for Corematal Eversion.

GLMM model used corematal eversion presence/absence as response variable, stimuli as the predictor variable, and individual moth ID as a random effect. Columns (C1-C5) are model contrasts between stimuli. Values in parentheses are standard errors on coefficient estimates.

	C1	C2	C3	C4	C5
(Intercept)	2.40***	0.33	−1.98***	−2.17***	−2.52***
	(0.39)	(0.32)	(0.36)	(0.42)	(0.50)
Tactile Stimulation		2.07***	4.38***	4.57***	4.92***
		(0.36)	(0.43)	(0.48)	(0.55)
Bat Echolocation	−2.07***		2.32***	2.50***	2.85***
	(0.36)		(0.32)	(0.39)	(0.47)
White Noise	−4.38***	−2.32***		0.18	0.54
	(0.43)	(0.32)		(0.40)	(0.47)
Female Conspecific	−4.57***	−2.50***	−0.18		0.35
	(0.48)	(0.39)	(0.40)		(0.52)
Male Conspecific	−4.92***	−2.85***	−0.54	−0.35	
	(0.55)	(0.47)	(0.47)	(0.52)	
AIC	560.42	560.42	560.42	560.42	560.42
BIC	586.99	586.99	586.99	586.99	586.99
Log Likelihood	-274.21	-274.21	-274.21	-274.21	-274.21
Num. obs.	620	620	620	620	620
Num. groups: Individual	31	31	31	31	31
Var: Individual (Intercept)	2.01	2.01	2.01	2.01	2.01

$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

Table 2. GC-MS results (Continued on Next Page). PEAK RT refers to the retention time of the most intense part of the HD peak, where HEIGHT is measured. AREA is the integration of intensity over the retention time window spanning START RT to END RT. All retention times are measured in minutes. See also: https://doi.org/10.6084/m9.figshare.4964270

SPECIES	INDIVIDUAL	TISSUE	PEAK RT	Height	Area	Start time	End time	HAS HD
HODAL 860uM	NA	NA	22.760	4619241.2	226191.5	22.5	24	YES
DCM BLANK	NA	NA	23.145	869.3	151.2	22.5	24	NO
E. zizana	1	COREMATA	22.892	100647.0	11581.7	22.5	24	YES
E. zizana	2	COREMATA	22.882	64037.4	5078.9	22.5	24	YES
E. zizana	2	BODY	22.871	2851.9	375.9	22.5	24	NO
E. zizana	3	COREMATA	22.871	31783.8	3799.2	22.5	24	YES
E. zizana	3	BODY	22.881	58036.9	12160.3	22.5	24	YES

Table 3. Putative PA compounds identified in three *E. zizana* males (Continued on Next Page). Dictionary masses come from Buckingham et al., 2010. Masses that exhibited multiple retention times were combined, but their retention times are reported. See Methods regarding “Platynecine NOX” and “Retronecine”. Mass measurements are given in amu. Retention time is given in minutes. See also: https://doi.org/10.6084/m9.figshare.4964270

Putative Compound	M+1 (Experimental)	Actual Mass (Experimental)	Actual Mass (Dictionary)	delta PPM	Retention Time (min)
Monocrotaline (Internal Standard)	326.1602	325.1529	325.1525	1.23	2.45
"Platynecine NOX"	174.1490	173.1417	173.1052	210.85	3.93
"Retronecine"	156.0770	155.0697	155.0946	160.55	0.47
Asperumine	320.1862	319.1789	319.1784	1.57	22.82
Callimorphine	298.1652	297.1579	297.1576	1.01	12.12
Callimorphine NOX	314.1602	313.1529	313.1525	1.07	14.02; 22.8
Compound 1 - Free Base	331.2236	330.2163	NF	NA	0.63
Compound 1 - NOX	347.2184	346.2111	NF	NA	0.62
Compound 2 - NOX	352.2336	351.2263	NF	NA	8.78; 14.38; 15.1; 16.17; 17.37; 17.88
Compound 3 - Free Base	346.2230	345.2157	NF	NA	20.97; 22.47
Compound 3 - NOX	362.2180	361.2107	NF	NA	22.73
Compound 4 - Free Base	348.2386	347.2313	NF	NA	14.08; 19.47; 20.6; 22.62
Compound 4 - NOX	364.2336	363.2263	NF	NA	11.35; 17.02
Curassavine	300.2173	299.2100	299.2097	1.00	10.85
Curassavine NOX	316.2099	315.2051	315.2046	1.38	9.3; 22.93
Heliocurassavicine	286.2015	285.1942	285.1940	0.70	19.87
Heliocurassavicine NOX	302.1961	301.1888	301.1889	0.25	10.37
Jacoline	370.1864	369.1791	369.1788	0.81	7.73
Leptanthine	318.1916	317.1843	317.1838	1.58	8.97; 14.93
Leptanthine NOX	334.1867	333.1794	333.1788	1.80	22.87
Lycopsamine	300.1811	299.1738	299.1733	1.67	14.15; 23.33
Platynecine	158.1177	157.1104	157.1103	0.44	0.53; 1.73; 2.9; 4.27; 6.62
Platynecine NOX	174.1127	173.1054	173.1052	1.16	0.6
Procerine	272.1495	271.1422	271.1420	0.74	5.83
Retronecine	156.0973	155.0950	155.0946	2.58	0.55
Retronecine NOX	172.0970	171.0897	171.0895	1.17	0.53
Strigosine	272.1861	271.1788	271.1784	1.29	10.1; 17.6
Strigosine NOX	288.1809	287.1736	NF	NA	14.65
Unknown 11	274.1652	273.1579	273.1576	1.10	5.62; 6.7; 11.15
Unknown 12	283.1181	282.1108	NF	NA	23.67
Unknown 13	294.1914	293.1841	NF	NA	11.83; 13.45; 14.17
Unknown 14	318.2280	317.2207	NF	NA	17.57; 18.65; 20.45
Unknown 15	322.2017	321.1944	NF	NA	23.27
Unknown 16	324.1424	323.1351	NF	NA	23.85
Unknown 17	328.2001	327.1928	NF	NA	18.13
Unknown 19	381.2239	380.2166	NF	NA	20.62; 21.33
Unknown 20	385.1649	384.1576	NF	NA	23.68
Unknown 3	216.1597	215.1524	NF	NA	18.8
Unknown 4	220.1468	219.1395	NF	NA	2.1
Unknown 5	227.1281	226.1208	NF	NA	20.65
Unknown 7	255.1231	254.1158	NF	NA	18.52
Unknown 9	267.1231	266.1158	NF	NA	23.87

Table 4. Concentrations (µM) and standard deviations for each compound (Continued on Next Page). Concentration values are given as µM with their standard deviations. Coremata from individual 3 was run 6 times. All other moth samples were run 7 times. Monocrotaline standard (MONO) was run 9 times. Blanks were run 23 times. Our internal standard, monocrotaline, is omitted from this table. See also: https://doi.org/10.6084/m9.figshare.4964270

			Individual 1	Individual 2		Individual 3	
Compound Area / ISTD Area (uM)	BLANK	MONO	Coremata	Body	Coremata	Body	Coremata
"Platynecine NOX"	0 ± 0	0 ± 0	63.8 ± 31.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
"Retronecine"	0 ± 0	0 ± 0	12 ± 3.5	19.8 ± 5	25.3 ± 7	6.8 ± 1.4	0 ± 0
Asperumine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.1	0 ± 0
Callimorphine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.8 ± 0.6	0 ± 0
Callimorphine NOX	0 ± 0	0 ± 0	109.5 ± 78.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Compound 1 - Free Base	0 ± 0	0 ± 0	0 ± 0	6.4 ± 2.5	0 ± 0	6.6 ± 2.7	0 ± 0
Compound 1 - NOX	0 ± 0	0 ± 0	0 ± 0	28.7 ± 16	0 ± 0	6.9 ± 4.5	0 ± 0
Compound 2 - NOX	0 ± 0	0 ± 0	77.2 ± 45.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Compound 3 - Free Base	0 ± 0	0 ± 0	12 ± 6.3	4.7 ± 3.1	2.1 ± 1.3	0 ± 0	2.2 ± 1.3
Compound 3 - NOX	0 ± 0	0 ± 0	0 ± 0	5.1 ± 3.1	3.9 ± 3.9	0 ± 0	0.6 ± 0.7
Compound 4 - Free Base	0 ± 0	0 ± 0	109.8 ± 65.4	6 ± 4.4	9 ± 4.4	0.5 ± 0.4	7.6 ± 5.8
Compound 4 - NOX	0 ± 0	0 ± 0	8.9 ± 5.7	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Curassavine	0 ± 0	0 ± 0	12.6 ± 8.7	0 ± 0	0.7 ± 0.5	0 ± 0	0 ± 0
Curassavine NOX	0 ± 0	0 ± 0	34.2 ± 44.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Heliocurassavicine	0 ± 0	0 ± 0	72.7 ± 25.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Heliocurassavicine NOX	0 ± 0	0 ± 0	2.6 ± 2.3	0.6 ± 0.4	0.3 ± 0.1	0 ± 0	0.6 ± 0.3
Jacoline	0 ± 0	0 ± 0	0 ± 0	53.5 ± 50.1	0 ± 0	5.1 ± 4.8	0 ± 0
Leptanthine	0 ± 0	0 ± 0	22.4 ± 6.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Leptanthine NOX	0 ± 0	0 ± 0	0.5 ± 0.2	0.6 ± 0.7	0.4 ± 0.5	0 ± 0	0 ± 0
Lycopsamine	0 ± 0	0 ± 0	90.8 ± 42.1	2.8 ± 0.7	1.4 ± 0.4	0 ± 0	2.3 ± 0.4
Platynecine	0 ± 0	0 ± 0	33.4 ± 18.7	178.1 ± 19.3	0 ± 0	231.5 ± 12.3	0 ± 0
Platynecine NOX	0 ± 0	0 ± 0	0 ± 0	134 ± 18.4	0 ± 0	73.8 ± 10.5	0 ± 0
Procerine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.3	0 ± 0
Retronecine	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0	0 ± 0	0.1 ± 0.1	0 ± 0
Retronecine NOX	0 ± 0	0 ± 0	0 ± 0	41.6 ± 7.9	0 ± 0	26.4 ± 3.2	0 ± 0
Strigosine	0 ± 0	0 ± 0	8.7 ± 4.4	0 ± 0	3.7 ± 2.8	0 ± 0	0 ± 0
Strigosine NOX	0 ± 0	0 ± 0	184 ± 80.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 11	0 ± 0	0 ± 0	2.6 ± 1.3	19.5 ± 0.6	0 ± 0	7.3 ± 0.2	0 ± 0
Unknown 12	0 ± 0	0 ± 0	375.9 ± 328.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 13	0 ± 0	0 ± 0	220.3 ± 82.1	0 ± 0	0 ± 0	0 ± 0	33.4 ± 14.2
Unknown 14	0 ± 0	0 ± 0	73 ± 46	11 ± 5.7	5.7 ± 5.4	0 ± 0	0 ± 0
Unknown 15	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.9 ± 0.5	0 ± 0
Unknown 16	0 ± 0	0 ± 0	50.5 ± 28	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 17	0 ± 0	0 ± 0	0 ± 0	3 ± 1	0 ± 0	0.9 ± 0.3	0.4 ± 0.1
Unknown 19	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 20	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 3	0 ± 0	0 ± 0	16.7 ± 8.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 4	0 ± 0	0 ± 0	10.1 ± 2.9	10.7 ± 0.7	0 ± 0	0 ± 0	0 ± 0
Unknown 5	0 ± 0	0 ± 0	13.5 ± 8.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 7	0 ± 0	0 ± 0	19.5 ± 7.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unknown 9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 5. Profile comparisons of tissue types between individuals. "B" refers to "Body" and "C" refers to "Coremata". Numbers correspond to the individual. UNIQUE denotes the number of compounds unique to a given tissue/individual. Intersection symbols denote shared compounds between indicted tissue/individual. PA COUNT are the total number of compounds in each tissue/individual. See also: https://doi.org/10.6084/m9.figshare.4964270

TABLE 5A	UNIQUE	B1 ∩ B2	B1 ∩ B3	B2 ∩ B3	B1 ∩ B2 ∩ B3	SUM	PA COUNT
B1	NA	NA	NA	NA	NA	NA	NA
B2	39%	0%	0%	61%	0%	100%	18
B3	27%	0%	0%	73%	0%	100%	15

TABLE 5B	UNIQUE	C1 ∩ C2	C1 ∩ C3	C2 ∩ C3	C1 ∩ C2 ∩ C3	SUM	PA COUNT
C1	63%	19%	4%	0%	15%	100%	27
C2	0%	50%	0%	10%	40%	100%	10
C3	14%	0%	14%	14%	57%	100%	7

Table 6. Profile comparisons of tissue types within individuals. Conventions follow Table 5. See also: https://doi.org/10.6084/m9.figshare.4964270

TABLE 6A	UNIQUE	B1 ∩ C1	SUM	PA COUNT
B1	NA	NA	NA	NA
C1	100%	0%	100%	27

TABLE 6B	UNIQUE	B2 ∩ C2	SUM	PA COUNT
B2	56%	44%	100%	18
C2	20%	80%	100%	10

TABLE 6C	UNIQUE	B3 ∩ C3	SUM	PA COUNT
B3	87%	13%	100%	15
C3	71%	29%	100%	7

CURRICULUM VITAE

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EDUCATION:

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2010 Research Assistant, PI: Dr. Wulfila Gronenberg, University of Arizona Department of Neuroscience

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RECENT PUBLICATIONS:

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2015 **Dowdy, N.J.** 2015. La Batalla Aérea en los Cielos de Ecuador. *Antorcha Verde*

2014 S. Perlman, **N. J. Dowdy**, L. R. Harris, M. Khalid, S. E. Kelly, M. S. Hunter. 2014. Factors Affecting the Strength of *Cardinium*-Induced Cytoplasmic Incompatibility in the Parasitic Wasp *Encarsia pergandiella* (Hymenoptera: Aphelinidae). *Microbial Ecology* **67**(3): 671-678.

TEACHING:

2017 Teaching Assistant, Parasitology (BIO321), Wake Forest University (WFU)

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2014 Teaching Assistant, Biology and the Human Condition (BIO101), WFU

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GRANTS / AWARDS:

2017 Entomological Collections Network Travel Grant -- $1,000
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2013 T. Roosevelt Research Grant, American Museum of Natural History -- $2,000
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CONFERENCE PRESENTATIONS / GUEST LECTURES / TALKS:

2016 Invited Talk, International Congress of Entomology 2016
"Different Strokes for Different Folks? Can we distinguish sonar-jamming and acoustic aposematism in tiger moths (Lepidoptera: Erebidae: Arctiinae)?"

2015 Invited Lecture, Animal Behavior, Wake Forest University
"Sensory and Behavioral Ecology of Bat Echolocation"

2015 15th International Meeting on Invertebrate Sound & Vibration
"*Acoustic Aposematism in Tiger Moths: Efficacy in the Field"*

2013 Neotropical Lepidoptera Course 2013, Jenaro Herrera, Peru
"All's Fair in Love and War: Moth Hearing and Sound Production for Anti-Predator Defense and Sexual Communication"

2012 North Carolina Natural Science Museum, Raleigh, NC, USA
"BATS, MOTHS, SCIENCE, AND YOU!"

2011 Graduate Seminar Series, Wake Forest University, Winston-Salem, NC, USA
"Biological Aerial Warfare"

2010 1st Annual Department of Ecology and Evolutionary Biology Poster Session (UA)
"Why Do My Younger Brothers Have Fewer Offspring?: A Story of Wasps and Bacteria"

2010 Annual UBRP Conference (UA)
"Odor Processing Centers Vary Across Species and Castes of Ants and Bees"

2010 Annual UBRP Conference (UA)
"Why Do My Younger Brothers Have Fewer Offspring?: A Story of Wasps and Bacteria"

2009 Annual UBRP Conference (UA)
"Competitive Influences of the Bacterial Symbionts Wolbochia and Cardinium on Encarsia inaron"

2009 Willow Canyon Physics Conference (Mt. Lemmon, Tucson, AZ, USA)
"Wing Beat Frequencies of Polistes flavus as a Function of Temperature"

UNDERGRADUATE MENTORING:

2017 **J. Clemmons, T. Scholfield-Johnson, K. Seymour, and P. Twyne**:
"Visual and acoustic aposematic signals of tiger moths (Erebidae: Arctiinae) do not depend on body size"
"Some relationships between visual and aposematic signals among tiger moths (Erebidae: Arctiinae)"

2016 **E. Metzler**:
"The Effect of Tymbal Morphology on Sound Produced in Tiger Moths (Erebidae: Arctiinae)"

2015 **T. Zhang**:
"Examining Tymbal Morphology of Palaearctic Tiger Moths (Erebidae: Arctiinae)"

2015 **L. Cansler:**
"Comparison of Microscopy Techniques for Tymbal 3-D Reconstruction"

2015 **L. Lazarchick**:
"Examining Tymbal Morphology of North American Tiger Moths (Erebidae: Arctiinae)"

2015 **M. Shaw**:
"Catch Me If You Can: The Effectiveness and Utility of Moth Sounds and Evasion Techniques"

2015 **C. Spell:**
"Sonar-jamming, Aposematism, or Both: Do Some Moth Sounds Serve Dual Functions?"

2014 **D. Guerrero**:
"Phenology, Distribution of Aposematic Tiger Moths (Erebidae: Arctiinae) in N. Carolina"

2014 **M. Spiewak:**
"Visualizing Sound in 3-D Using Microphone Arrays and 3-D Videography"

2014 **T. Hoskins**:
"Utilizing Molecular Tools to Detect Tiger Moth (Erebidae: Arctiinae) DNA in Bat Guano"

2014 **R. Koski-Vacirca**:
"Automated Analysis of Bat and Moth Sonar"

2013 **D. Margius**:
"Effectiveness of Acoustic Aposematism in the Field"

2012 **K. Roman**:
"Bats Jamming Bats? A Playback Experiment"

2011 **Z. Walker**:
"Defending Food Patches by Jamming Sonar"

SERVICE:

2017	Committee for Information Technology, Wake Forest University, Elected Graduate Student Representative
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OUTREACH:

2016	Lead Presenter / Guide, Moth Week, Prairie Ridge Ecostation, Raleigh, NC, USA
2014	Lead Presenter / Guide, Family Safari Night, Prairie Ridge Ecostation, Raleigh, NC, USA
2013	Lead Presenter / Guide, Bat Night at Prairie Ridge Ecostation, Raleigh, NC, USA
2012	BugFest 2012 Booth: BUGS VS. BATS!, North Carolina Natural Science Museum, Raleigh, NC, USA

CONSULTING:

2015	Wildlife and Filming Consultant, BBC Production "*Skyworlds*"
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