ABSTRACT

MOTHS FIGHT BACK: ARMS RACE IN THE CLOUD FOREST

by

Diana Pamela Rivera Parra

Moths and bats engage in a coevolutionary arm race, where the same signals bats use to find moths are being used by moths to avoid bats. Moths evolve not only behavioral but acoustic responses to avoid predation by bats. This research examines one small component of a complex, multispecies arm race between moths and bats. In this study we found that the moth Amastus hyalina displayed both flight and acoustic responses to ultrasonic stimuli.

In tethered flight the tiger moth *Amastus hyalina* (Arctiinae) exhibits a complex array of reactions to ultrasonic tones that includes changes in flight and production of ultrasonic clicks. The changes in flight included change in wing beat frequency, amplitude of the stroke, a rotation of the wings, and deflection of the abdomen, legs, and antennae. The changes in flight displayed by moths reduce the cues that bats use for prey capture including the amplitude modulation of echoes from wingbeats. Moths produce the biggest returning echoes for frequencies between 20 to 35 kHz, which coincides with the frequencies used by most insectivorous bats in the location. The species of tiger moth that we studied is but one of many species at our field site, but we believe that similar antipredator mechanisms are widespread among tiger moths, and may also be found in other families of moths that have tympanic organs.

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by
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CHAPTER 1

INTRODUCTION

1.1 Predator Prey Interactions

In the arms race between predators and their prey, prey often perform behaviors that reduce the likelihood of capture. Prey have two main adaptations of behaviors to avert predation: predator avoidance or anti-predator behaviors (Brodie Jr *et al*., 1991; Ferrari *et al*., 2011). Predator avoidance are behavioral mechanisms that reduce the probability of encountering the predator by temporal avoidance, spatial avoidance, or crypsis (Soutar & Fullard, 2004; Blumstein, 2006). Anti-predator mechanisms, on the other hand, reduce the probability of a successful predation attack (Brodie Jr *et al*., 1991; Soutar & Fullard, 2004). These include deterrent signals, defensive structures, aposematism, mimicry, startle behavior, and temporal distraction among others. Whether an animal uses one or more of these strategies are the result of evolutionary processes and depends on the idiosyncrasies of their natural and evolutionary histories.

Insectivorous bats and moths are a well-known and experimentally tractable example of such a complex predator/prey relationship. Insectivorous micropteran bats are crepuscular or nocturnal feeders and use ultrasonic signals to find and identify insect prey. The main cues used by these bats are acoustic features of the returning echoes from moths. As a result of selection pressure from ultrasonic bats, moths have evolved several anti-predator mechanisms, many of which depend on the detection of echolocation calls produced by bats. I am interested in this arms race, especially in relation to biodiversity of bats and moths in Ecuador: there can be over 100 species of bats in a single hectare in Ecuador and thousands of species of moths.

1.2 Evolutionary History

Moths and bats are entangled in a coevolutionary arms race, where the ultrasonic calls produced by bats for the localization and characterization of moth prey are being used by moths to detect the predatory bats and avoid them. Perhaps the most important adaptation in moths is the appearance of tympanic organs that are specialized for the detection of ultrasonic signals, allowing them to hear incoming bats (Roeder, 1974; Hoy, 1992; Rydell *et al*., 1995; Miller & Surlykke, 2001; Yager, 2012). Indeed, this adaptation likely permitted the subsequent emergence of a large number of anti-predator strategies.

But why are moths active at night? If moths simply avoided flying at night, would they not avoid predation by echolocating bats? A great diversification event of Lepidoptera (moths and butterflies) happened around 100 million year ago, which coincided in time with the origins of the major angiosperm clades (Miller & Surlykke, 2001; de Jong, 2007; Wahlberg *et al*., 2013). At that time there were many species of insectivorous birds, which are almost exclusively diurnal, but nocturnal bats had not yet evolved. As a result, the nighttime skies were apparently free of predators such as bats; so nocturnal Lepidoptera could exploit nocturnal flowering plants in a predator free environment (de Jong, 2007; Wahlberg *et al*., 2013).

At present, the great majority of species of moths are most active after dusk and through the night, where they are actively looking for food, mates, and oviposition sites (Acharya, 1995*a*). Moths feed on nectar from flowering plants, they have specific oviposition sites since the larvae will feed on the host plant, and they use pheromones for long-range sexual communication. The female emits pheromones either while flying, or while stationary once it has found the host plant. The males are generally more active flyers than females, as they are searching for and following plumes of pheromones emitted by females (Acharya, 1995; New, 2004).

How did echolocation evolve in bats? A great ecological diversification of bat species occurred after the evolution of flight and echolocation. These species evolved from an arboreal, gliding, insectivorous mammal that used short, low intensity, broadband tonal signals only for spatial orientation (Schnitzler *et al*., 2003; Jones & Teeling, 2006; Simmons *et al*., 2008; Conner & Corcoran, 2012).

The evolution of flight is thought derive from jumping, to gliding, to finally flying. Echolocation is believed to have first been used for obstacle avoidance and spatial orientation. Bats likely only used prey-generated sounds to detect and localize insect prey. Later echolocation mechanisms became refined -- first for perch hunting and subsequently for continuous aerial hawking, where bats actively looked for insects using echolocation systems (Schnitzler *et al*., 2003; Jones & Teeling, 2006; Simmons *et al*., 2008).

There are several lines of evidence that support the idea that moth tympanic organs evolved to detect echolocating bats. First, the tuning of the tympanic organs corresponds to the frequencies of most insectivorous bat calls (Waters & Jones, 1996; Jones & Waters, 2000; ter Hofstede *et al*., 2013). Second, there is a positive correlation between auditory sensitivity and how active are moths at night with more active flyers exhibiting greater sensitivity (ter Hofstede *et al*., 2008; Nakano *et al*., 2015). Third, ultrasound elicits anti-predator behaviors such as evasive maneuvers, acoustic defenses, and combinations of different behaviors (Corcoran *et al*., 2009*b*; Conner & Corcoran, 2012; Nakano *et al*., 2015).

In contrast, earless moths rely in predator avoidance mechanisms to reduce the probability of encountering bats. They have more erratic flight patterns; prefer to fly in areas close to the ground or areas where bats normally don't hunt. Moths can also use temporal avoidance by means of seasonal isolation such as being active before bats emerge from hibernation, being active before dust. Finally, they have reduced flight activity and are cryptic (Greenfield & Weber, 2000; Soutar, 2004). There is a general trend that earless moths tend to be larger and heavier, making them not a suitable prey for small insectivorous bats (Fullard & Napoleone, 2001).

Moths in the family Noctuidea possess two tympanic organs, one on each side of the metathorax (Waters, 2003; Yager, 2012; Conner & Corcoran, 2012). In response to ultrasound, they dramatically alter their behavior with aerobatic evasions that include loops, dives, flight cessation, and different mechanisms to attain acoustic concealment (Jones & Waters, 2000; Miller & Surlykke, 2001; ter Hofstede *et al*., 2008; Conner & Corcoran, 2012). A subfamily of this group, Actiinae, known as tiger moths, have gone a step further and have tymbal organs that are used to produce ultrasonic jamming signals in response to bat echolocation calls (Surlykke & Miller, 1985; Corcoran *et al*., 2009*a*; Corcoran & Conner, 2012). Their metathoracic tymbal organs produce sound through cuticular buckling, which produce two burst of clicks as the tymbal buckle inward and outward. The number of clicks they produce changes on each species depending on the number of striations found on the tymbals (Barber & Conner, 2006*a*; Corcoran *et al*., 2010; Corcoran & Hristov, 2014).

Tiger moths are just one of many species of insects that produce sounds in response to hearing ultrasound: a species of geometrid moth has prothoracic tymbal organs, tiger beetles produce sounds by beating of hind-wings against their elytra, hawkmoths produce sound by genital stridulation, and caterpillars of saturniidae moths are able to produce sound by mandibular tooth strikes. Other species of moths are also able to produce ultrasonic clicks, which are used for intraspecific communication (Acharya & Fenton, 1999; Greenfield & Weber, 2000; Rodríguez-Loeches *et al*., 2009).

1.3 How Insectivorous Bats Find Moths

Insectivorous bats use echolocation to localize and characterize moths (Griffin *et al*., 1960; Surlykke, 1988; Schnitzler & Kalko, 2001). The frequency of echolocation calls is specific to each species of bat and ranges between 8 kHz to 215 kHz (Schnitzler & Kalko, 2001). Echolocation calls are not only used in prey capture, but are also used for navigation at night, the characterization and localization of other objects in the environment, and in social communication (O'Farrell M. J., Miller, 1997; Schnitzler & Kalko, 2001; Jung *et al*., 2014).

The temporal and spectral patterns of ultrasonic calls often vary in relation to the behavioral task. For example, bats alter the temporal and spectral features of calls during three different phases of a predatory strike on a prey. The first phase is searching, during which the bat produces calls at a lower rate with relatively long and constant intervals. After detection of a moth, they enter the approach phase, during which they produce calls with increasingly shorter durations and shorter interpulse intervals. The final phase is known as the terminal or feeding buzz, during which calls are produced at a high repetition rate with a reduced bandwidth and lower frequencies (Miller & Surlykke, 2001; Schnitzler & Kalko, 2001).

Bats listen for changes in the returning echoes from the wings and bodies of moths (Roeder, 1974; Kober, 1990; Moss & Zagaeski, 1994). Specifically, bats commonly use the modulation of acoustic features of echolocation calls by moth wings. Movements of the moth cause an amplitude modulation that depends on the position of the wings relative to the angle of the wings (Roeder, 1974; Kober, 1990; Moss & Zagaeski, 1994). The point where the wings are nearer to the top of the stroke is the most favorable moment for the bat to retrieve information from the echoes (Roeder, 1974). The wings of the moth act as an acoustic mirror where there is an optimal reflection position for the returning signal, which produces an acoustical glint. The echo spectra of a flying moth present amplitude modulation in rhythm of the wingbeat (Roeder, 1974; Kober, 1990). Most moths have wingbeat frequencies of 10 to 40 beats per second, and the acoustical glint is restricted to the short moment when the moving wing is in an optimal position for reflection (Schnitzler *et al*., 1983). When a bat has identified a moth in open space, it change its call from search calls to approach calls with an increase in its pulse repetition rate (Roeder, 1974); which will give more information to the bat and higher chances to hit the moth at the optimal position for reflection.

To cope with the various antipredator mechanisms of moths, bats likewise have evolved different echolocation strategies. One strategy is to use high or low allotonic frequencies, meaning they use echolocation calls outside the main frequency sensitivity of the tympanic organ of the moth (20-60 kHz; Schoeman & Jacobs, 2003). Another strategy is the one used by gleaners, which are bats that take prey from substrate. Their echolocation calls are low intensity, or they stop echolocating and use acoustic cues from their insect prey to localize them (Ratcliffe & Fullard, 2005).

1.4 How Moths Avoid Bats

Many moths are able to detect bat echolocation calls (Miller & Surlykke, 2001; Conner & Corcoran, 2012). The ability to detect the signals provides them the opportunity to generate behaviors that reduce the likelihood of predation by bats (Schoeman & Jacobs, 2003; Yager, 2012; Conner & Corcoran, 2012). Most moths are sensitive to ranges of 20 to 60 kHz, although other species are able to detect frequencies up to 100 kHz (Waters & Jones, 1996; Jones & Waters, 2000; ter Hofstede *et al*., 2013). Moth hearing is quite sensitive, detecting signals with amplitudes as low as 25-45 dB SPL. Given that echolocation calls can be as loud as 120db at 10cm (Surlykke, A., Kalko, 2008), moths are able to detect an oncoming bat at distances of more than 20m (Yager, 2012). Considering the flight speed of both animals, a moth has about 1s response time available after detecting an echolocating bat (Yager, 2012).

1.4.1 Behavioral Mechanisms

Moths have a bimodal anti-bat behavior (Hoy & Robert, 1996; Miller & Surlykke, 2001; Ratcliffe *et al*., 2009). If the moth detects a search call of a bat at a distance, the moth will fly away in a controlled, directional flight. However, if the moth detects an approach call of a bat that is close, the moth will fly in an erratic pattern or even interrupt it's flying (Miller & Surlykke, 2001; Conner & Corcoran, 2012). Cessation of flight movements and closure of the wings can eliminate the major source of echoes, as well as the amplitude modulation produced by flapping, providing the insect with some measure of acoustic concealment as it falls to the ground (Schnitzler *et al*., 1983).

1.4.2 Acoustic Mechanisms

Many tiger moths (Actiinae) produce ultrasonic clicks in response to echolocation calls (Corcoran *et al*., 2009*a*; Conner & Corcoran, 2012). Three main defense mechanisms have been attributed to the clicks, which are also correlated with the presence or absence of defensive chemicals (Barber & Conner, 2006*b*; Corcoran *et al*., 2010). These are 1) acoustic aposematism (low clicking rate and presence of toxic chemicals; Barber & Conner, 2006*b*; Corcoran *et al*., 2010); 2) acoustic mimicry (low clicking rates and no presence of chemicals; Barber & Conner, 2006*b*; Corcoran *et al*., 2010); and 3) sonar jamming (high clicking rate and no presence of chemicals; Barber & Conner, 2006*b*; Corcoran *et al*., 2010, 2011).

The timing at which the moths produce clicks seems to play an important role if information interference is the mechanism being use. Therefore, moths that present a low click rate and start clicking early on the ongoing attack are thought to be using the clicks as an aposematic signal. While a high click rate and clicking later on the ongoing attack is related with jamming, this is hypothesized to work in two ways. Either by producing multiple targets which is the phantom-echo hypothesis, or by disrupting the echo processing, which is the interference hypothesis (Barber & Conner, 2006*b*; Corcoran *et al*., 2011; Conner & Corcoran, 2012). Since prey-generated sounds are the cues that bats use to extract the necessary discriminatory information from the clicks (Barber *et al*.,

2009) click rate is proposed as the main variable to determine the underlying function of the clicks.

1.5 Behavioral or Acoustic Mechanisms, Do Moths Have to Choose Only One?

For this research we capture the tiger moth *Amastus hyalina* (Erebidae-Arctiinae), which displayed click production and changes in flight in response to ultrasonic tones while in tethered flight. These moths produce ultrasonic clicks that can be coupled with changes in flight. The changes in flight are a multiple component behavior that includes changes in wings kinematics, and the positions of the head, legs, abdomen, and wings. This moth lives in the cloud forest in Ecuador, where it faces the predation pressure of more than 30 species of insectivorous bats. Even though it is sympatric with hundreds of other species of moths and other insects, this moth has evolved a set of two responses to ultrasound, which were previously thought to be mutually exclusive (Corcoran *et al*., 2010; Corcoran & Hristov, 2014).

What exactly are the predation pressures *Amastus hyalina* faces? Is the array of responses used by these tiger moths solely to avoid bats, or do they have an intraspecific function such as mating calls? The changes in flight and click production must first be discussed separately to understand their implications in the acoustic world where they interact with bats. Observations of animals in tethered flight give us the opportunity to analyze the changes in kinematics (Yager *et al*., 1990), and to search for the link between click production and flight changes.

As a step toward understanding bat/moth interactions, I have focused my research efforts on the anti-predator behaviors in moths, specifically in relation to the 1) detection of ultrasonic signals from bats, and 2) the production of ultrasonic jamming signals and flight behaviors in moths. What is the spectrum of calls that moths can detect, and how do moths avoid predation using combinations of ultrasonic signals and changes in the characteristics of flight behaviors?

CHAPTER 2

MATERIALS AND METHODS

2.1 Field Site and Animal Collection

This research was conducted in the eastern cloud forest of Ecuador, at Yanayacu Biological Station and Center for Creative Studies. Moths were collected during 12 consecutive nights in July of 2015 using black light traps.

Each night the traps were set up around the station, and moths were captured until midnight and kept in a plastic container until used in experiments during that night. Every moth was sexed by visual inspection, photographed, and identified before each experiment; all animals were released at the end of the night. Research was conducted under the permit (N 21-IC-FAU-DPAN/MA) of the Ministry of Environment of Ecuador and in collaboration with the “Pontificia Universidad Católica del Ecuador”.

2.2 Experimental Setup

Experiments were conducted inside a custom made flight tunnel that was illuminated with infrared lights. The flight tunnel consisted of three separate parts the intake funnel with a flow-straitening honeycomb, the experimental section, and an end section where the fan was located. It was made of extruded metal poles and acrylic panels (80/20 Inc. Indiana, United States). The entire flight tunnel measured 2.50 meters. High-speed video and sound recordings (described below) were made throughout each experiment (Figure 2.1).

Figure 2.1 Experimental setup. (A) High-speed camera, (B) Speaker, (C) Microphone, (D) Infrared lights, (E) 1401 data acquisition system, and (F) Computer.

Source: Photo taken by Andrea Roeser, 2015.

Moths were fixed to an entomological pin on its pronotum (structure that covers the thorax of insects) with a drop of melted wax. The position of the moth was maintained throughout the experiments at around 17cm from the speaker, given the measurements of the experimental chamber. The position of the speaker was changed in relation to the moth during the experiments: we used three positions including lateral, ventral, and dorsal to the moth. Experiments were started only after the moth was flying continuously for at least one minute.

Tone stimuli consisted of three 1-second tones with 2 seconds of silence in between. Frequencies of the stimuli ranged from 10 to 100 kHz in ranges of 5 kHz (Table 1). Six different pre-recorded bat calls were also used as stimuli. The order of stimulus type, position, and intensity was randomized.

Table 2.1 List of randomized ultrasonic tones used as stimuli, frequency in kilohertz

Tone number	1st	2nd	3rd
1	40	10	40
2	10	95	35
3	45	15	25
4	100	35	70
5	20	50	85
6	60	30	95
7	55	90	40
8	80	65	75

2.3 Video and Sound Settings

The video recordings were made with a high-speed camera, Mega Speed PRO X7 (Mega Speed Corp, San Jose, California, United States). Videos were shot at 1200 frames per second with an image size of 680x480 pixels. The camera was mounted above the tunnel using a tripod, and moths were filmed from the rostro-doral aspect through the top panel.

A Petterson L400 ultrasound speaker (Pettersson Elektronik AB, Uppsala, Sweden) was used for the playbacks of the acoustic stimuli. Tones were digitally generated using MATLAB. Bat calls from the field site were also used as stimuli. For recording the bat calls, an AR180 ultrasonic receiver (Binary Acoustic Technology LLC, Tucson, Arizona, United States) was used, which has an operating range of 1-180 kHz. The echolocation calls of bats were recorded and saved using SPECT'R software (Binary Acoustic Technology LLC, Tucson, Arizona, United States), which was in auto mode.

For the ultrasonic recordings of moth clicks, a custom made microphone system was used. The microphone was connected to a 1401 data acquisition system (Cambridge Electronic Design Limited, Cambridge, England), and files were saved using Spike v.8.2 software (Cambridge Electronic Design Limited, Cambridge, England). The microphone was located above the speaker to record the returning echoes from the moth.

2.4 Data Analysis

The videos were analyzed using custom MATLAB programs. The wingtips were tracked using standard approaches for automatic detection of movement (Figure 2.2). For this experiment the wings were only tracked in two dimensions. We extracted the wingbeat frequency and amplitude of the stroke. We also analyzed the amplitude and frequency modulations of the returning echoes recorded by the microphone.

Figure 2.2 Example of the wingtips tracking. Green corresponds to the left wing, red corresponds to the right wing.

For change in wingbeat frequency, amplitude of the stroke, and wing echo a univariate analysis of the variance was done using SPSS v.17 statistical software for Windows (IBM SPSS Statistics, United States) with an alpha of 0.5. A separate analysis for each parameter measured was done in relation to each variable: frequency of the stimuli, amplitude of the stimuli, and position of the speaker in relation to the moth. Finally, for the click production a chi squared test was done for males and females, and for each of the three variables: frequency of the stimuli, amplitude of the stimuli, and position of the speaker in relation to the moth.

CHAPTER 3

RESULTS

3.1 General Description

A total of 30 moths from the species *Amastus hyalina* (Erebidae-Arctiinae; Figure 3.1) were captured (15 males and 15 females) from which a total of 650 videos were recorded. Each video was composed of up to three experimental trials. For each individual trial we obtained a video sequence of five seconds and an audio file containing the recording of the returning echo from the moth. All of the trials where the moth was not flying before the tone, did not have a consistent flight, or the wings move unevenly were not used in the analyses.

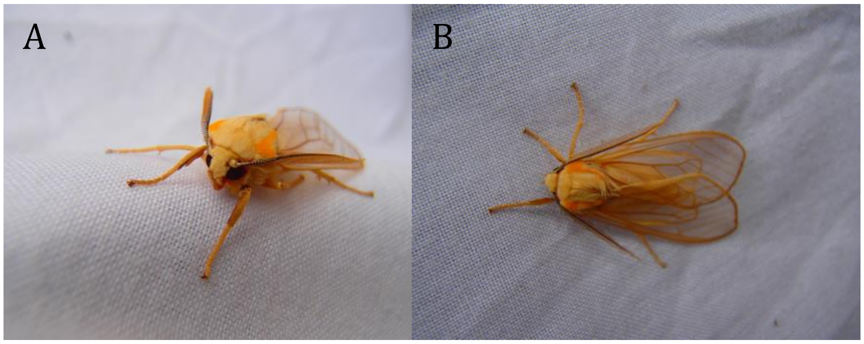

Figure 3.1 Pictures of a male of *Amastus hyalina* (Erebidae-Arctiinae). (A) Frontal view. (B) Dorsal view.
Source: Photo taken by Andrea Roeser, 2015.

In general females flew more consistently than males. To ensure the moths maintained sustained flight trough the trials a fan in low speed was used on all trials. In some cases were moths would not start flying spontaneously we would increase fan speed for a moment in order to try to elicit flight before starting the experiment. There was no reaction to just the fan, and no obvious noise from the fan was recorded. We did not notice habituation to either the wind stimuli used to elicit flight behavior nor to the acoustic stimuli.

3.2 Flight Response to Ultrasound

3.2.1 Normal Flight

Moths had a wingbeat frequencies of around 30 Hz (25-32 Hz), with amplitude of the stroke that was different for each individual. In sustained flight adopted a body position with the abdomen down in the horizontal plane and the legs tucked in close to the body. The head was maintained straight in relation to the body with the antennae hold at midrise (Figure 3.2A).

We did not observe behavioral differences between males and females during sustained flight. This species of moth is monomorphic: males and females are the same size, and have the same coloration, except for some red spots on the pronotum scales of the males. However we notice one difference in the activity pattern between both sexes. During the first hours of sampling we captured more females than males, with an increasing number of males coming to the light later on. This could indicate that females become active first, perhaps to find oviposition sites and produce pheromones only after the males become active.

3.2.2 Flight Changes

Moths produced a broad spectrum of flight responses to ultrasound, which we divide into three main categories. In the first category, moths stopped flying in response to the stimuli and did not restart flight even after the stimuli was over. This category of reaction was mostly observed with bat calls playbacks. In the second category, moths presented a continuous flight, there was no other behavioral components observed in reaction to the stimuli. In the third category, moths presented a momentary pause in flight in response to ultrasound but never stopped flying. This response was characterized by extension of the legs, deflection of the abdomen, and flexion of the antennas (Figure 3.2B). There were also changes in the wings which included changes in wingbeat frequency, change in amplitude of the stroke, and a slight anterior rotation of the wings. Given the methodology used in the experiments, the components described for the category three of reaction were only noted and no measurements were possible.

Figure 3.2 Behavioral responses to ultrasound, side view and frontal view. (A) Stable flight. Note the position of the abdomen, and legs close to the body. (B) Same individual after presentation of an ultrasonic tone. Note foreleg extension, antennae and abdomen deflection.

3.3 Changes in Wing Kinematics

We tracked the movement of the tips of the wings in two axes, from where we extracted wingbeat frequency and amplitude of the stroke. For both parameters we calculate the percentage of change before the stimuli and during the stimuli.

There was a significant difference for the different tone frequencies used as stimuli for both wingbeat frequency and amplitude of the stroke ($p<0.05$, Table A.1 and A.2). For the position of the speaker relative to the moth (top, bottom, and side) there was a significant difference for wingbeat frequency but not for the amplitude of the stroke ($p<0.05$, Table A.3 and A.4). Finally, there was no significant difference for wingbeat frequency and amplitude of the stroke for the amplitude of the stimuli used (3-low, 5-high, $p>0.05$, Appendix). Both males and females presented the highest change in wingbeat frequency and wingbeat amplitude to stimuli frequencies between 20 to 60 kHz (Figure 3.3).

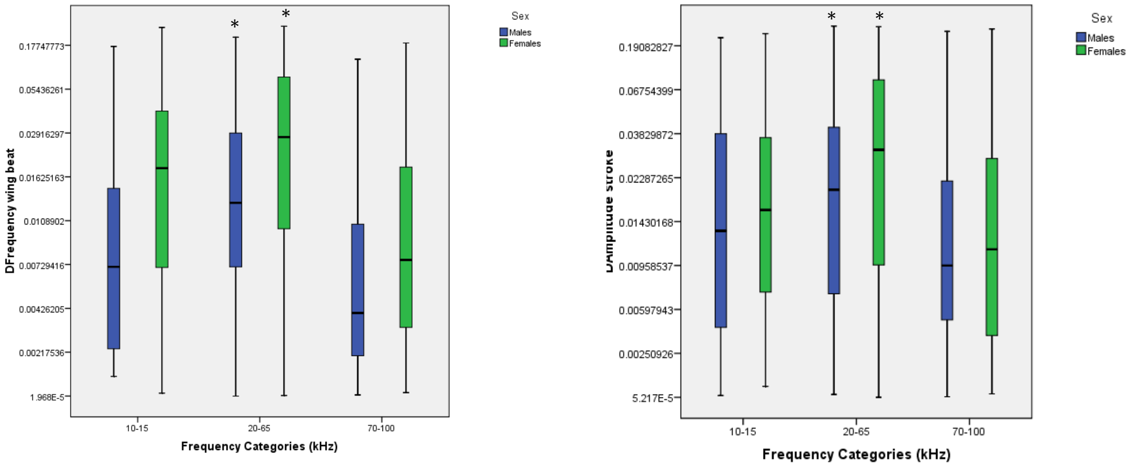

Figure 3.3 Whisker plot of the changes in the frequency of the wingbeat (left panel) and amplitude of the stroke (right panel) in response to ultrasound for the different stimuli frequencies. Both males and females presented the greatest changes for stimuli frequencies of 20 to 65 kHz ($p<0.05$).

There was also a significant difference between males and females in wing kinematics. We found that females changed the amplitude and frequency of wingbeats more than males when challenged with ultrasonic tones (Figure 3.3, $p<0.05$, Table A.5 and A.6).

3.4 Changes in the Returning Echoes

We measured the changes in the returning echo of the stimuli, which was recorded with a microphone located above the speaker. The returning echo was characterized by amplitude modulations related to the wingbeats. We found that moths' had the strongest echoes for stimulation frequencies between 20 to 35 kHz (Figure 3.4, $p<0.05$, Table A.7). There was also a change in the amplitude modulation of the returning echo when moths displayed changes in flight in response to ultrasound. There was a reduction of the amplitude modulation of the returning echo during the display of changes in body posture and changes in flight (Figure 3.6, 3.7).

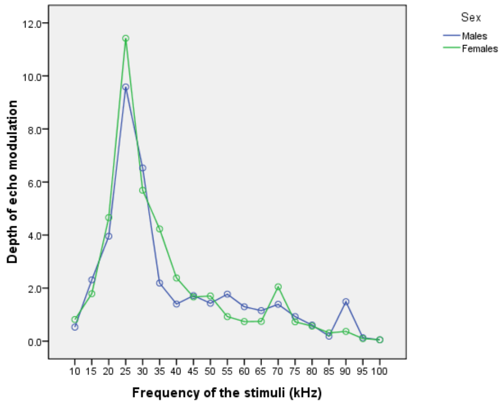

Figure 3.4 Change in the depth of the echo modulation for males and females at all the frequencies use as stimuli. The strongest echoes is for stimulation frequencies between 20 to 30 kHz.

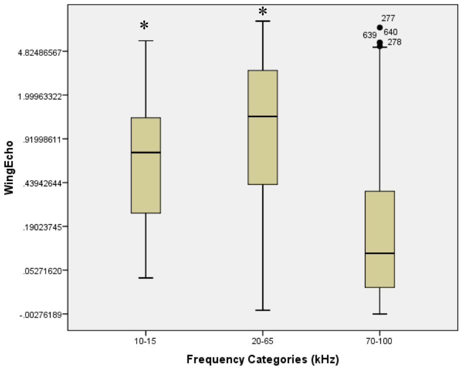

Figure 3.5 Whisker plot for changes in the depth of the echo modulation for males and females at all the frequencies use as stimuli. The strongest echoes is for stimulation frequencies between 20 to 30 kHz ($p<0.05$).

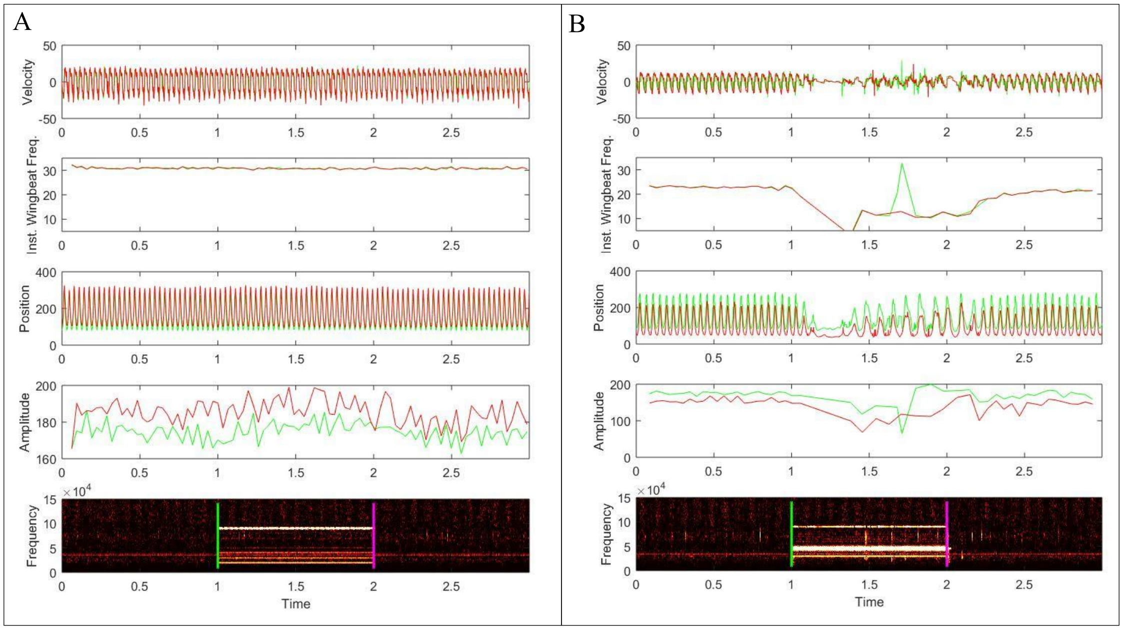

Figure 3.6 Example of changes in the kinematic parameters of the wings when a moth presented changes in flight in response to an ultrasonic tone. (A) Example of a case where a moth did not presented changes in flight in response to ultrasound. (B) Example of a case where a moth presented changes in flight to response to ultrasound. Panels from the bottom to the top represent: sonogram of the sound channel recording, information about the tone and the returning echo, amplitude of the stroke, position of the wingtip, instantaneous wingbeat frequency, velocity of the wing tip. Note the production of clicks in panel B.

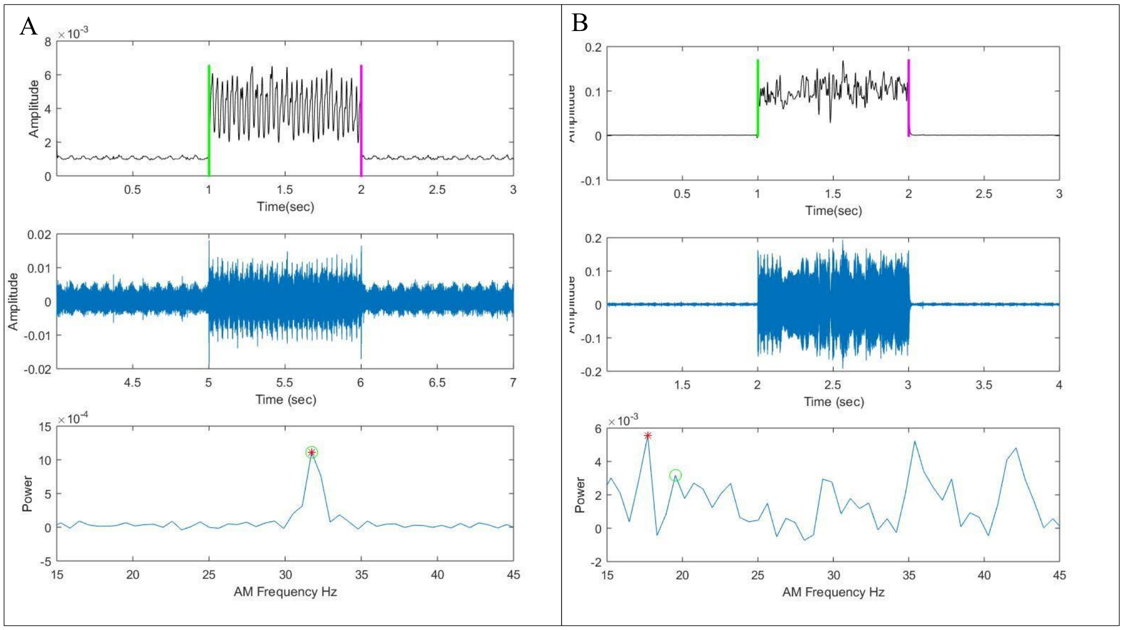

Figure 3.7 Example of the change in the depth of the amplitude modulation of the echo a moth presented changes in flight. (A) Example of a case where a moth did not presented changes in flight in response to ultrasound. (B) Example of a case where a moth presented changes in flight to response to ultrasound. Panels from bottom to the top represent: spectrum of the envelope, waveform of the raw signal, amplitude envelope.

3.5 Acoustic Response to Ultrasound

In response to ultrasound both males and females produce ultrasonic clicks. The number of clicks varied greatly between individuals and trials, whit as few as one click and as much as 50 clicks being produced. The clicks were frequency-modulated signals, with the main frequency around 40kHz, and a broadband of 10-90kHz. There was a significant difference between males and females, with more females producing clicks than males (p=0.000, Table A.8), and both males and females produced more clicks in stimuli frequencies between 20 to 65 kHz and when the speaker was located lateral to the moth (Figure 3.8, p=0.000, Table A.9 and A.10), the two different amplitudes of the stimuli used did have an effect (Table A.11).

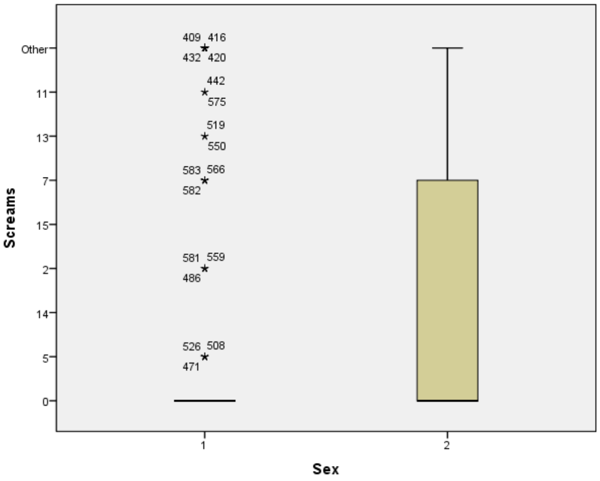

Figure 3.8 Number of clicks produced in males and females. There were more females that produce clicks than males (p=0.000).

CHAPTER 4

DISCUSSION

4.1 General Discussion

The tiger moth *Amastus hyalina* (Erebidae-Arctiinae) presented an array of responses to ultrasound which include changes in flight and production of ultrasonic clicks. These set of responses has an effect on the amplitude modulation of the returning echo of the signal, which is the main cue used by bats for prey capture.

Moths typically did not react to ultrasound when they were not flying, possibly due to the fact that the tympanic organ is located under the wings and during flight these hearing structures are exposed, but when the moth is at rest the tympanic organ is covered by the wings. Further data support this idea since in some cases, when the moth was not flying but the wings were spread dorsally, the moths reacted to acoustic stimuli and even started flying (data not shown). Another reason could be that the anti-predator mechanisms are specific for aerial insectivorous bats, which hunt them while in flight, so once the moths are at rest, they can count on predator avoidance strategies such as crypsis.

4.2 Acoustic Response

In response to ultrasound both males and females produce ultrasonic clicks. The number of clicks varied greatly between individuals and trials, whit as few as one click and as much as 50 clicks being produced. The mechanism by which this species of moth produce the clicks is still unknown, and we believe it could be using a different mechanisms from previously described tymbal organs in other tiger moths. The reason is that in previously described tiger moths click production a double burst of clicks is observed, as the tymbal buckle inward and outward (Barber & Conner, 2006*b*; Corcoran *et al*., 2009*b*). The number of clicks they produce depends on the number of striations found on the tymbals (Barber & Conner, 2006*a*; Corcoran *et al*., 2010; Corcoran & Hristov, 2014). We observed as few as one click and as much as 50 in one case, but we did not observe the double burst of clicks.

Determining the mechanism by which moths' ultrasonic clicks work has proven to be challenging. Moths' clicks have been generally considered as a defense mechanism that is interfering with the echolocation system of bats, three main hypothesis have been formulated about how clicks work which are: clicks startle the bat, jam the echolocation system of the bats, or work as an aposematic signal —which is related with the fact that some tiger moths are toxic— (Fullard et al., 1994; Corcoran et al., 2009b, 2010). We do not know if this moth *Amastus hyalina* is toxic, it does not present bright colors as other tiger moths, and the only visual aposematic signal we saw is the production a yellow liquid from the sides of the pronotum after manipulation. Therefore we can hypothesize that the clicks they produce are aposematic signals and have an antipredator function since they were produce in response to ultrasound, but we cannot draw any further conclusions until further experiments are done with bats.

4.3 Changes in Flight

This specie of moth when presented with ultrasonic tones displayed three main categories of changes in flight. In one of the categories the reaction was accompanied by general changes in the body posture and appendages. The moths extended their legs, starting in some cases with an extension of the back legs as soon as the stimuli started, and a full extension of all 6 legs later on during the stimuli. They also deflected the abdomen, and flex the antennas. In relation to the wings there was a change in wingbeat frequency and wingbeat amplitude. Interestingly moths also presented a slight anterior rotation of the wings. Since all of our experiments were done in individuals in tethered flight we cannot know the exact effects these changes in posture of the different parts of the body have in terms of flight. But they are accompanied with changes in the wing kinematics which suggest that these changes in posture could be an orchestrated maneuver when in free flight. If we think in flight dynamics a change in body posture can alter the center of mass relative to the center of lift and thrust produced by the wings (Dyhr *et al*., 2013).

The changes observed in the posture in legs, abdomen, and antenna could play a stabilizing role during free flight based on results published for other insects. The extension of the legs in bees has been showed influence aerodynamic torques, it increases the moment of inertia by decreasing roll acceleration, and also increases overall body drag (Combes & Dudley, 2009). The movement of the abdomen probably contributes to general stability serving as a brake to strong pitch torques generated by wings (Hinterwirth & Daniel, 2010). In hawk moths movements of the abdomen contribute to pitch stability during flight, these assist in the transition between stable flight to hovering to maneuvers (Dyhr *et al*., 2013). Interestingly moths' antennas have been described to have a similar role in conveying information about rotation of the animal as halteres do in Diptera (Sane *et al*., 2007; Hinterwirth & Daniel, 2010).

As for the changes observed in the wings (change in frequency wingbeat, amplitude of the stroke, and a rotation of the wings) they all have aerodynamic effects. The wingstroke of an insect is divided into four kinematic portions, two translational phases (upstroke and dowstroke), and two rotational phases (pronation and supination; Dickinson *et al*., 1993, 1999). The changes in wingbeat frequency and amplitude of the stroke have a direct effect on the translational phase. While the observed rotation of the wings probably has an effect in the rotational phase, which contributes to the lift and plays an important role in modulating the direction and magnitude during steering maneuvers (Combes & Dudley, 2009; Hinterwirth & Daniel, 2010).

Both the changes observed in the wings and the posture change in legs, abdomen, and antenna can be translated in changes that lead to a controlled maneuver that based in our results are performed in response to ultrasound. The changes in posture has been previously described in praying mantis (Yager & May, 1990), and tiger beetles (Yager & Spangler, 1997). The study using tiger beetles involved tethered flight, whereas the study using mantids was in both non-flying animals and in free flying individuals (Yager *et al*., 1990). Free flying male mantises exhibited three categories of responses to ultrasonic signals. When faced with distant sounds, mantids reacted with level turns away from the sound source with no obvious change in flight speed. When presented with nearby sounds, mantids reacted with diving turns or with more extreme spiral dives. In the case of the spiral dives, the mantis abruptly goes into a steep power dive that takes it to the ground during which there is no active control of the trajectory and the mantis does not flap its wings. In contrast, spiral dives are actively controlled with a continue wingbeat, changes in yaw and pitch, and control of roll (Yager *et al*., 1990). Potentially these spiral dives described in mantis could be the same maneuvers we would observed in free flight on the moth *Amastus hyalina* in reaction to insectivorous bat calls.

4.4 Acoustical Meaning of the Reaction to Ultrasound

The information encoded in the returning echoes has to be considered in two components, one in which the amplitude of the echo changes when the moths presented changes in flight, and the second one that the moths reflect the biggest echoes for frequencies between 20 to 35 kHz. Both types of changes in the echo are relevant for the insectivorous bats hunting strategies.

Bats analyze the returning echo to find flying insects, detecting both amplitude and frequency modulations (Roeder, 1974; O'Neill & Suga, 1982; Moss & Zagaeski, 1994). The moth *Amastus hyalina* alter the temporal and spectral features of the amplitude and frequency of echoes via changes in their flight behavior, which may offer a form of concealment from bat echolocation systems. The rotation of the wings we observed could potentially contribute to this reduction in the amplitude modulation of the returning echo. Zeng *et al*. (2011) found that the scales of the moths increase absorbance of ultrasound (Zeng *et al*., 2011), this could be potentially be part of the reason why moths rotate their wings. By rotating their wings more surface area of the wings is exposed during the downstroke, and possibly reduces the strength of the returning echo. The rotation of the wings, and maybe even the changes in posture of the different parts of the body, could be part of a concealment mechanism to escape from insectivorous bats.

Interestingly this species of moth produced the largest amplitude echoes at stimulation frequencies between 20 to 35 kHz, which coincide with the frequencies being used by most insectivorous bats in the location. It is clear that these species of moth are likely to be acoustically conspicuous for the bats, but this feature must be considered in the context of the diversity of species of moths and anti-predator behaviors in this habitat.

Further experiments with different species of moths in this habitat will be interesting as they may reveal how these different mechanisms may improve bat avoidance across species. Consider that a truly randomized response to bat calls would be the best possible strategy, as bats could not predict or learn how a moth might respond to its calls. If each of the many species of moths used a different strategy, the result would potentially be, from the perspective of the bat, highly randomized responses by prey. In other words, even though the response of a particular species of moth to bat calls might be predictable, if many species use different responses, the result is that the bat would not be able to predict the behavior of moth prey.

4.5 Females vs. Males

We found that females changed the amplitude and frequency of wingbeats more than males when challenged with ultrasonic tones. If we consider the biology of moths, males are generally more exposed to predation since they fly more than females. Female moths fly to find the host plant and produce pheromones to attract males to the oviposition site, whereas males fly to encounter pheromone plumes to find the females. Males spend more time flying as they search for and follow pheromone plumes.

Nevertheless our results for this species of moth show that females are more responsive to ultrasound than males, which would make males potentially more susceptible to predation. In fact previous work suggest that males are more frequently captured than females (Acharya, 1995). Why do males are less reactive if they are more exposed to predation? Maybe there is a significant cost for males to alter their flight behavior when they are following a pheromone plume, they may lose track of the female. Or it could be that the tympanic organ of the males is less sensitive than the females.

4.6 Conclusions

These results examine one small component of a complex, multispecies arm race between moths and bats. In this study we found that the moth *Amastus hyalina* displayed both flight and acoustic responses to ultrasonic stimuli. Moths clearly can differentiate between bat calls and tones. The changes in flight displayed by moths reduce the cues that bats use for prey capture including the amplitude modulation of echoes from wingbeats. Moths produce the biggest returning echoes for frequencies between 20 to 35 kHz, which coincides with the frequencies used by most insectivorous bats in the location. The species of tiger moth that we studied is but one of many species at our field site, but we believe that similar antipredator mechanisms are widespread among tiger moths, and may also be found in other families of moths that have tympanic organs. Similar behavioral responses have been described in mantis and tiger beetles (Yager & May, 1990; Yager & Spangler, 1997) but an analysis of the effects of this behavior on the acoustics of the signal has not been analyzed.

4.7 Future Directions

Further experiments with free flying bats and moths would help us make observations of the anti-predator mechanisms. Observe how the bats react to the different mechanisms that the moths' posses would help us to further understand the acoustic meaning of these defenses. Precise recordings of in vivo interactions would further give us information about predation and survival rate.

Experiments with different species of moths are needed, to investigate if this acoustic concealment strategy is wide spread. Especially experiments with species that present differences in the amount of scales in their body, to further investigate the idea that the scales of the moths absorb ultrasound (Zeng *et al*., 2011).

There are a number of variables to further investigate upon this "new" strategy here described. The use of a moth robot could be a useful tool for a more detailed manipulation of the variables to further understand the acoustic change in the signal. It would allow us to investigate different species of moths, and how does the absorbance change in different angles of the wings toward the sound source, among others.

Further research in intraspecific communication is needed, since this moth presented different click production mechanisms than the one previously described for other tiger moths. We believe that these differences could be mainly because of the array of response they have that they don't have to rely solemnly on the acoustic defense. But there could be a secondary use of the clicks, where they don't need such an elaborate and refine click production mechanisms.

Experiments for male's reaction to ultrasound in the presence of pheromones and neural recordings of their tympanic organ could help us elucidate if males are in fact less reactive, whether is because of the presence of pheromones or because their tympanic organ is less sensitive than the female's.

Finally, a multi-approach experimentation is needed, to fully understand the population dynamic in such a biodiversity place. If hundreds of species of moths are flying at the same time, how strong is the predation pressure on each species or individuals that they have evolved such intricate mechanisms. Studies with components of neuroethology would contribute knowledge to how the nervous circuits in the moth control such diverse mechanisms.

APPENDIX

STATICALLY ANALYSIS RESULTS

Table A.1 Results of the univariate analysis of variance for change in wingbeat frequency in relation to the 20 different stimuli frequencies. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change Wingbeat Frequency

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.041	1	.041	9.581	.006	.320
	Error	.088	20.397	.004[a]			
Freq	Hypothesis	.253	18	.014	3.230	.008	.764
	Error	.078	18	.004[b]			
Sex * Freq	Hypothesis	.078	18	.004	1.104	.343	.030
	Error	2.530	641	.004[c]			

a. .933 MS(Sex * Freq) + .067 MS(Error), b. MS(Sex * Freq), c. MS(Error)

Table A.2 Results of the univariate analysis of variance for change in amplitude of the stroke in relation to the 20 different stimuli frequencies. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in Amplitude of the stroke

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.023	1	.023	4.442	.047	.172
	Error	.112	21.451	.005[a]			
Freq	Hypothesis	.217	18	.012	2.343	.040	.701
	Error	.092	18	.005[b]			
Sex * Freq	Hypothesis	.092	18	.005	.777	.729	.021
	Error	4.238	641	.007[c]			

a. .933 MS(Sex * Freq) + .067 MS(Error), b. MS(Sex * Freq), c. MS(Error)

Table A.3 Results of the univariate analysis of variance for change in wing beat frequency in relation to the three different positions of the speaker relative to the moth. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in the wingbeat frequency

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.068	1	.068	194.371	.000	.899
	Error	.008	21.718	.000[a]			
Pos	Hypothesis	.013	2	.006	56.534	.017	.983
	Error	.000	2	.000[b]			
Sex * Pos	Hypothesis	.000	2	.000	.026	.975	.000
	Error	2.900	673	.004[c]			

a. .943 MS(Sex * Pos) + .057 MS(Error), b. MS(Sex * Pos), c. MS(Error)

Table A.4 Results of the univariate analysis of variance for change in amplitude of the stroke in relation to the three different positions of the speaker relative to the moth. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in the amplitude of the stroke

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.008	1	.008	.454	.567	.179
	Error	.037	2.088	.018[a]			
Pos	Hypothesis	.013	2	.006	.338	.747	.253
	Error	.037	2	.019[b]			
Sex * Pos	Hypothesis	.037	2	.019	2.753	.064	.008
	Error	4.529	673	.007[c]			

a. .943 MS(Sex * Pos) + .057 MS(Error), b. MS(Sex * Pos), c. MS(Error)

Table A.5 Results of the univariate analysis of variance for change in wing beat frequency in relation to the two amplitudes of the stimuli used. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in the wingbeat frequency

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.062	1	.062	7.686	.220	.885
	Error	.008	1	.008[a]			
Amp	Hypothesis	.027	1	.027	3.337	.319	.769
	Error	.008	1	.008[a]			
Sex * Amp	Hypothesis	.008	1	.008	1.910	.167	.003
	Error	2.870	675	.004[b]			

a. MS(Sex * Amp), b. MS(Error)

Table A.6 Results of the univariate analysis of variance for change amplitude of the stroke in relation to the two amplitudes of the stimuli used. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in the amplitude of the stroke

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	.013	1	.013	.594	.582	.373
	Error	.022	1	.022[a]			
Amp	Hypothesis	.006	1	.006	.299	.681	.230
	Error	.022	1	.022[a]			
Sex * Amp	Hypothesis	.022	1	.022	3.207	.074	.005
	Error	4.544	675	.007[b]			

a. MS(Sex * Amp), b. MS(Error)

Table A.7 Results of the univariate analysis of variance for change in amplitude of returning echo in relation to the 20 different stimuli frequencies used. Alpha .05.

Tests of Between-Subjects Effects

Dependent Variable: Change in the Wing Echo

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Sex	Hypothesis	2.166	1	2.166	.276	.604	.010
	Error	218.427	27.814	7.853[a]			
Freq	Hypothesis	4148.442	18	230.469	34.081	.000	.971
	Error	121.723	18	6.762[b]			
Sex * Freq	Hypothesis	121.723	18	6.762	.292	.998	.008
	Error	14838.627	641	23.149[c]			

a. .933 MS(Sex * Freq) + .067 MS(Error), b. MS(Sex * Freq), c. MS(Error)

Table A.8 Results of the Chi-Square Test for click production between males and females.

Test Statistics

	Sex	Screams
Chi-Square	24.853[a]	125.388[a]
df	1	1
Asymp. Sig.	.000	.000

a. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 340.0.

Table A.9 Results of the Chi-Square Test for click production in relation to the 20 different stimuli frequencies used.

Test Statistics

	Screams	Freq
Chi-Square	125.388[a]	57.200[b]
df	1	18
Asymp. Sig.	.000	.000

a. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 340.0. b. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 35.8.

Table A.10 Results of the Chi-Square Test for click production in relation to the two different stimuli amplitudes used.

Test Statistics

	Screams	Amp
Chi-Square	125.388[a]	1.153[a]
df	1	1
Asymp. Sig.	.000	.283

a. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 340.0.

Table A.11 Results of the Chi-Square Test for click production in relation to the two different stimuli amplitudes used.

Test Statistics

	Screams	Pos
Chi-Square	125.388[a]	92.941[b]
df	1	2
Asymp. Sig.	.000	.000

a. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 340.0. b. 0 cells (.0%) have expected frequencies less than 5. The minimum expected cell frequency is 226.7.

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