The bat–moth arms race has existed for over 60 million y, with moths evolving ultrasonically sensitive ears and ultrasound-producing organs to combat bat predation. The evolution of these defenses has never been thoroughly examined because of limitations in simultaneously conducting behavioral and phylogenetic analyses across an entire group. Hawkmoths include >1,500 species worldwide, some of which produce ultrasound using genital stridulatory structures. However, the function and evolution of this behavior remain largely unknown. We built a comprehensive behavioral dataset of hawkmoth hearing and ultrasonic reply to sonar attack using high-throughput field assays. Nearly half of the species tested (57 of 124 species) produced ultrasound to tactile stimulation or playback of bat echolocation attack. To test the function of ultrasound, we pitted big brown bats (Eptesicus fuscus) against hawkmoths over multiple nights and show that hawkmoths jam bat sonar. Ultrasound production was immediately and consistently effective at thwarting attack and bats regularly performed catching behavior without capturing moths. We also constructed a fossil-calibrated, multigene phylogeny to study the evolutionary history and divergence times of these antibat strategies across the entire family. We show that ultrasound production arose in multiple groups, starting in the late Oligocene (~26 Ma) after the emergence of insectivorous bats. Sonar jamming and bat-detecting ears arose twice, independently, in the Miocene (18–14 Ma) either from earless hawkmoths that produced ultrasound in response to physical contact only, or from species that did not respond to touch or bat echolocation attack.

Significance

Ultrasound production is one of the most sophisticated antibat strategies in nocturnal insects, yet it has never been thoroughly studied in a phylogenetic framework. We conducted high-throughput field assays using playback of echolocation attack sequences, laboratory bat–moth interaction experiments, and fossil-calibrated phylogenetic analyses to provide the first evidence that multiple unrelated hawkmoth species produce ultrasound and jam bat echolocation. Our robust tree demonstrates that sonar jamming evolved twice during the Miocene after the radiation of insectivorous bats. We provide an example of the power behind collaborative science for revealing the function and historic pattern of behavior, and predict that ultrasound production is a widespread antibat strategy in the extraordinary diversity of nocturnal insects.
material from the same moths to build a fossil-calibrated, multigene molecular phylogeny of hawkmoths. Our behavioral experiments and phylogenetic analyses illustrate, for the first time to our knowledge, the function and evolution of antibat ultrasound production in a diverse group of insects.

Results and Discussion

We present a comprehensive behavioral dataset and couple it with the most complete and robust phylogeny of hawkmoths to date, to reveal a fascinating history of bat–moth interactions. After testing the ultrasound response of 124 hawkmoth species to tactile handling and playback of multiple frequency-modulated bat echolocation attack recordings (Dataset S1), we found that males of nearly all species in the Acherontiini s.l., Ambulycini, and Macroglossinae generated broadband, primarily ultrasonic sounds with their genitalia (Fig. 1 B–D). Surprisingly, all three groups have a strikingly different modified scale patch on the inner surface of their genital valves and the stridulation mechanism is unique to each (Movies S1–S3). Some of these ultrasound-producing species lack ears (e.g., Ambulycini, Dilophonotini, and lower Macroglossini), implying that ears are not a prerequisite for sound production. Nearly all species in the Acherontiini s.l. and Choerocampina produced an ultrasonic reply to bat echolocation, confirming the pioneering work of Roeder et al. (29) and Göpfert and Wasserthal (30). Hawkmoths that have ears did not respond acoustically to echolocation when their palps were removed, a result consistent with Roeder and Treat (31) and Roeder et al. (32), who showed that the acoustic sensitivity of Choerocampina decreases dramatically after amputation of the labial palp. Some female hawkmoths also produced ultrasound (Dataset S1), but because our sampling of females was very limited, these data were consequently not incorporated into the ancestral state reconstruction analyses.

We pit big brown bats (Eptesicus fuscus; Vespertilionidae) against hawkmoths in two experiments to test the function of hawkmoth antibat ultrasound. In our first experiment, an adult E. fuscus was exposed to individuals of the falcon sphinx moth (Xylophanes falco) for four consecutive nights, and a second bat was similarly tested for eight nights. Recent diet analyses reveal that E. fuscus consistently preys on Lepidoptera from a variety of families (33), so this was an appropriate bat species for these experiments. Each night, two sound-producing, palatable X. falco were randomly presented along with eight other palatable, silent moths: two X. falco with their sound-producing genital structures ablated, two white-lined sphinx moths (Hyles lineata; a naturally silent hawkmoth, similar in size to X. falco, included as a negative control for prey body size), and four silent, palatable, female greater wax moths (Galleria mellonella). Logistic regression analysis showed that all three types of silent moths were significantly more likely to be captured than sound-producing X. falco: ablated X. falco were 33.0-times [95% confidence interval (CI) = 2.9–374.4] more likely to be captured, H. lineata were 11.0-times [95% CI = 1.4–85.1] more likely, and G. mellonella were 121.0-times [95% CI = 6.7–2188.4] more likely (Fig. 2A). The probability of capture did not significantly vary between different nights, indicating that bats did not habituate to the moths’ acoustic defense and that the moth’s sound production was

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**Fig. 1.** Antibat ultrasound in three hawkmoth lineages. (A) ML phylogenetic tree of Sphingidae simplified to show relationships among major groups. Numbers by branches are ML bootstrap support values and Bayesian posterior probabilities; numbers after each taxon name indicate the known species diversity according to hawkmoth classification (18). (B) Lateral view of the left male genital valve showing ultrasound-producing scales, anterior to the left. (Scale bar, 1 mm.) (C) Close-up view of stridulatory scales, anterior to the left. (Scale bar, 500 μm.) (D) Spectrograms (frequency in kilohertz on the y axis and time in milliseconds on the x axis; FFT 256) and power spectra (frequency in kilohertz on the y axis and sound intensity in decibels on the x axis; FFT 256) of modulation cycles produced by species from each of the three lineages; (Top to Bottom) Ambulyx pryeri (Ambulycini), Psilogramma discostriga (Acherontiini), Xylophanes falco (Choerocampina). In all cases, two bursts of sound are created as the valves scrape dorsomedially across the abdominal file, then after a short pause the valves scrape ventrolaterally across the file as they return to a resting position. See Movies S1–S3 for structural movement of valves. *Psilogramma* is here treated as part of the broader Acherontiini.

**Fig. 2.** Results of bat–moth interaction experiments. (A) Male X. falco and (B) male and female X. tersa are significantly less likely to be captured than hawkmoths with sound-producing structures removed or control moths. Ablated X. tersa data (n = 4) were collected during noncontiguous trials with the same bats. (C) Big brown bats (E. fuscus) show similar echolocation attacks, as measured by interpulse interval patterns, when attacking sound-producing and silent moths (also see Fig. S8). Spectrograms of ultrasonic response to playback of a frequency-modulated sonar attack for taxa from the two groups thought to jam bat sonar: Theretra rhesus (D) and Psilogramma discostriga (E). A blue triangle indicates a hawkmoth antibat modulation cycle.
immediately and consistently effective. Bats that failed to capture sound-producing hawkmoths often performed catching behavior without subduing prey (Movie S4).

The second experiment was conducted on naive bats to control for potential previous experience with sound-producing prey. Each evening, for four consecutive nights, bats were exposed to two sound-producing *Xylophanes tersa* males, two sound-producing *X. tersa* females, and four silent female *G. mellonella* controls. Logistic regression analysis showed that the odds of a silent control moth being captured were 16.7-times (95% CI = 1.3–100.0) greater than the odds of an *X. tersa* male being captured and 25.0-times (95% CI = 2.1–100.0) greater than the odds of an *X. tersa* female being captured (Fig. 2F). As in the first experiment, hawkmoth ultrasound production (in both male and female moths) was immediately and consistently effective at thwarting attack and bats regularly performed catching behavior when failing to capture moths. We examined sonar emissions and found that bats, in both experiments, attacked all silent control and sound-producing moths with comparable echolocation patterns (Fig. 2C), similarly progressing through the approach, track, and terminal phases of attack (34).

Results of these multnight experiments are not congruent with either a warning or startle function and instead are consistent with a jamming role for hawkmoth ultrasound production. Two bats avoided the first sound-producing hawkmoth they experienced, suggesting that startle may function ephemerally, as has been shown in tiger moths (12). However, we view it as unlikely that short-lived startle behavior is maintaining and driving the evolution of sound production in bat–moth interactions. In addition, two points further support that hawkmoths are not warning of bad taste: (i) bats consumed all ablated and control hawkmoths that were captured; and (ii) we conducted palatability experiments on three continents with 26 species of distantly related hawkmoths and 5 different bat species, and in all cases all hawkmoths tested were completely consumed (Table S1).

The only moth previously known to definitively jam sonar, *Bertholdia trigona* (Erebidae: Arctiinae), has a duty cycle of ~44% (14). In contrast, *Cynicia tenera*, a tiger moth that may possess limited jamming ability, has a duty cycle of ~8% (8, 12), and the sound-producing tiger moth *Euchaites egle*, which appears to be unable to jam sonar, has a duty cycle of only ~3% (12). Duty cycle, or sound per unit time, is likely related to jamming efficacy (35, 36). *X. tersa* and *X. falco* have respective duty cycles of ~18% and ~27%. It seems likely that antibat jamming is a continuum, with higher duty cycles being more effective at confusing predators. Based on the results of our bat–moth experiments, we estimated that duty cycles of ~20% or higher are capable of jamming bat sonar in species that we have not tested in bat–moth interactions.

We examined our behavioral findings in an evolutionary context with a new, multigene molecular phylogeny. Many relationships that were previously challenging to estimate were resolved. Our maximum-likelihood (ML) and Bayesian consensus trees were topologically near identical, resulting in well-resolved phylogenetic relationships that were previously challenging to estimate were resolved. The origin of hawkmoth ultrasound production took place soon after the origin of tiger moths, the latter of which arose in the late Eocene (39). Many tiger moths are chemically defended because bats likely make prey selection decisions during aeroacoustic maneuvering that can span a second or more.

An additional selective pressure that cannot be discounted is sex. Male tiger moths are known to use ultrasound for mate attraction (48). Although there have been no definitive empirical data confirming that hawkmoths use ultrasound during mating, Mell (49) observed, on two occasions, a male hawkmoth (*Psilogramma menephron*) stridulating while circling above a female. For several hawkmoth species tested, both sexes used their genitalia to produce ultrasound (Dataset S1). We thus predict that some hawkmoths use ultrasound during mating and that sexual selection likely plays a role in the maintenance and evolution of sound production.

In this study, we simultaneously investigated proximate and ultimate questions of animal behavior (50), via laboratory and field experiments, to begin uncovering the historic patterns of antibat strategies in a long-standing arms race. We predict that ultrasound production is widespread in the extraordinary diversity of nocturnal insects, mediated by aposematic, mimetic (both Batesian and Müllerian), and jamming mechanisms.

**Materials and Methods**

**Taxon Sampling.** Hawkmoths were sampled at 70 sites in 32 countries (Dataset S2). We conducted field-based echolocation playback behavioral experiments to 124 species before storing tissues of each specimen in 100% ethanol for eventual inclusion in the phylogenetic analysis. This integrated approach enabled us to obtain behavioral and molecular data from the same specimens to better examine the evolution of antibat behaviors. Voucher specimens of all moths are stored in the collection of the Florida Museum of Natural History, University of Florida; acoustic files are archived at the Cornell Lab of Ornithology Macaulay Library (Accession no. 3884). New DNA sequence data are available from GenBank (Accession nos. KP719983–KP720300),

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datasets and accompanying files are available from the Dryad Data Repository (www.datadryad.org; Accession Number 10.5061/dryad.3450r).

High-Throughput Behavioral Assays. We tethered 734 moths in free flight at the end of a 5-mm-diameter hollow plastic rod; a monofilament line was tied between the thorax and abdomen, threaded through the rod, and held securely while the flying moth was queried for acoustic response to tactile stimulation and playback of bat attacks. We examined acoustic responses in several tethering scenarios and elected to use the above method, as it did not interfere with sound production. To record moth sounds, we used Avisoft UltraSoundGate 116Hn hardware (sampling at 250 kHz onto a laptop computer running Avisoft Recorder software) and a CM16 condenser microphone (±3 dB, 20–140 kHz) positioned 10 cm from the posterior end of the moth’s abdomen (location of the sound-producing structures) and presented moths with three echolocation attack sequences from a speaker (Avisoft UltraSoundGate Player BL Pro-2, ±9 dB, 18–100 kHz) placed 10 cm from the head, perpendicular to the length of the moth body (Supporting Information).

We measured moth signals using Avisoft SASLab Pro and defined each cycle of the stridulatory apparatus as a modulation cycle. All signal parameters were computed from three modulation cycles per individual. We used responses from tactile trials to characterize signals to prevent corruption from overlapping bat sounds in the echolocation playback trials. To determine if moth sounds were spectrally or temporally different when produced during tactile or playback trials, we examined several modulation cycles from multiple species and found no differences. We measured temporal parameters from the oscillogram and spectral values from power spectra (FFT 1024, 50% overlap). To calculate duty cycle of the moth sounds, we counted the number of clicks that occurred in 100 ms, multiplied this by the average click duration of the modulation cycle (both measured using the Pulse Train Analysis tool in SASlab Pro), and divided this value by 100. We used this approach to allow for a direct comparison with tiger moth acoustic analyses (14, 35). Duty cycles, peak frequencies, and bandwidths (defined as the difference between the upper and lower frequency that is ±15 dB from the peak frequency) are presented in Table S2.

Behavioral Laboratory Experiments. We conducted all vertebrate care in accordance with Boise State University’s Animal Care and Use Committee’s guidelines (IACUC #006-AC11-015). We collected big brown bats (E. fuscus) as adults or preflight juveniles at colonies in Idaho, and maintained them on a diet of mealworms (larvae of Tenebrio molitor) and greater wax moths (G. mellonella) along with vitamin supplements. We performed experiments in an anechoic foam-lined indoor flight facility at Boise State University (7.6 m × 6.7 m × 3 m). During each experimental presentation of a flying moth tethered to a fine monofilament line, bats were allowed to hunt for 1 min. To establish a positive control, E. fuscus bats were trained to hunt silent, palatable, female G. mellonella; this hunting behavior was carefully observed to ensure that all bats used in these experiments were similar in their effectiveness at capturing moths. To capture each bat-moth interaction, we used three digital, high-speed, infrared-sensitive video cameras (Basler Scout, 120 frames per second) and Maxtraq3D software (Innovison Systems), and a desktop computer. We illuminated the interaction space with eight infrared Wildlife Engineering LED arrays and recorded ultrasound with a four-channel Avisoft UltraSoundGate 416H (sampling at 375 kHz) and four CM16 condenser microphones (±3 dB(Z), 20–140 kHz), using the Avisoft Recorder software described above. We calculated interpulse intervals (the temporal gap between sonar pulses) of echolocation attacks in Avisoft SASLab Pro.
Molecular Dataset Construction. The molecular dataset included five nuclear loci and one mitochondrial gene, totaling 7,449 bp, building on an available dataset of hawkmoth phylogenies. Outgroups were selected based on previous phylogenetic analyses of Bombycoidea (51, 52). Genes and their sequence lengths were: pyrimidine biosynthesis (CAD; 2,928 bp), dopa-decarboxylase (DDC; 1,282 bp), elongation factor-1α (EF-1α; 1,228 bp), Period (PER; 951 bp), wingless (WG; 402 bp), and cytochrome oxidase I (COI; 658 bp). The majority of sequence data were generated at the University of Maryland, College Park, MD, using RT-PCR, for which the primers, protocols, and molecular techniques are outlined in recent publications (e.g., refs. 37, 43, 51, and 53). Individual gene datasets were initially created to test for laboratory contamination, and subsequently concatenated into a single data matrix. For further details, see Supporting Information.

Alignment, Partitioning, and Phylogenetic Methods. All genes were concatenated and aligned with MAFFT v7.037 (54). The most optimal partitioning strategy and model of evolution was identified through PartitionFinder v1.0.1 (55). ML analyses were run in RAxML v7.3.2 with 1,000 tree searches with a backbone topological constraint (56). The Bayesian analysis was run in MrBayes v3.2 (57) with one cold chain and three hot chains, also implementing a backbone constraint. Converged MrBayes runs were combined after the deletion of burn-in and a majority rule consensus tree was created. In all analyses, trees were rooted with Macrothylacia rubi (Lasiocampidae) following recent studies that confidently placed Lasiocampidae as the sister family to remaining bombycooids (43, 51, 52, 58). Further details on the implementation of all phylogenetic methods are found in Supplementary Information.

Divergence Time Estimation. To estimate divergence times, we used fossil calibration data from Parham et al. (59). We also used a calibration on the root node of Sphingidae following the interfamilial Lepidoptera dating analysis of Wahlberg et al. (39). We used a Bayesian phylogenetic relaxed molecular-clock model with four calibration points to conduct a divergence time analysis in BEAST 1.7.5 (60). Branch rates were estimated with an uncorrelated relaxed clock model, allowing for the rate of evolution to vary among the branches of the tree with no prior correlation (Brownian motion) on a per lineage and per branch basis. Parameters were unlinked across partitions, and a Yule tree prior was used, which assumes a constant per lineage selection rate. We used the tree with the highest likelihood from the RAxML analysis and rescaled the tree in r8s 1.8 (61). We used the r8s ultrametric tree as the starting tree for all BEAST runs.

Two fossil-age estimates were based on a lognormal distribution, allowing us to assume that the actual divergence event took place before the earliest appearance of that fossil. For the third fossil age estimate, we used a uniform distribution based on morphological dissections that sound production was coded as present or absent for two different categories, based on whether mosfs made ultrasound in response to bats and touch. To determine ancestral states of these discrete categories, we ran the analyses in a ML framework in Mesquite (66) and BayesTraits (67), using the RAxML tree (Supporting Information). We mapped duty cycle as a continuous character on the ML phylogeny using the contMap function of Phytools v0.4-67 (68) in R v3.1.0 (69). Duty cycle values were available for 92 taxa in the tree (45.5%), and data for the remaining taxa and their ancestral states were estimated using likelihood by applying the ancML function in contMap.


