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Anti-bat ultrasound production in moths is globally and phylogenetically widespread

Jesse R. Barber[a,1], David Plotkin[b], Juliette J. Rubin[b,c], Nicholas T. Homziak[b], Brian C. Leavell[a,2], Peter R. Houlihan[b,c,3,4], Krystie A. Miner[a], Jesse W. Breinholt[b,d], Brandt Quirk-Royal[a], Pablo Sebastián Padrón[b,e], Matias Nunez[f,g,h], and Akito Y. Kawahara[b,1]

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Warning signals are well known in the visual system, but rare in other modalities. Some moths produce ultrasonic sounds to warn bats of noxious taste or to mimic unpalatable models. Here, we report results from a long-term study across the globe, assaying moth response to playback of bat echolocation. We tested 252 genera, spanning most families of large-bodied moths, and document anti-bat ultrasound production in 52 genera, with eight subfamily origins described. Based on acoustic analysis of ultrasonic emissions and palatability experiments with bats, it seems that acoustic warning and mimicry are the raison d'être for sound production in most moths. However, some moths use high-duty-cycle ultrasound capable of jamming bat sonar. In fact, we find preliminary evidence of independent origins of sonar jamming in at least six subfamilies. Palatability data indicate that jamming and warning are not mutually exclusive strategies. To explore the possible organization of anti-bat warning sounds into acoustic mimicry rings, we intensively studied a community of moths in Ecuador and, using machine-learning approaches, found five distinct acoustic clusters. While these data represent an early understanding of acoustic aposematism and mimicry across this megadiverse insect order, it is likely that ultrasonically signaling moths comprise one of the largest mimicry complexes on earth.

acoustic mimicry | antipredator defense | biodiversity

Significance

Moths have been evading echolocating bat attack for tens of millions of years. Here, we show that the ultrasonic defensive sounds of moths are, in fact, widespread across the planet and the tree of life. Our decade-long investigation reveals that perhaps 20% of moths produce anti-bat sounds, with at least 6 independent origins of sonar-jamming behavior and more than 10 origins of acoustic aposematism (warning of noxious taste). The sheer number of lepidopterans that produce ultrasonic warning sounds indicates that moth acoustic emissions might create the most diverse mimicry rings (groups of species that converge on similar warning signals) on Earth. Comprehending this ultrasonic symphony is central to understanding insect biodiversity.

Across systems, unpalatable prey declare their location and identity to predators (1). Gaudy poison frogs and red newts alert attackers of toxins sequestered in their skin glands (2, 3), brightly banded coral snakes warn birds of their venomous bite (4), and patterned milkweed bugs and monarch butterflies proclaim their unpalatable hemolymph (5). While aposematism [conspicuous signaling to advertise noxiousness (6)] has been most rigorously studied in the visual system, warning displays have also been described in the olfactory (7) and auditory systems (8). Until now, acoustic aposematism has appeared as either an accessory in a multisensory warning suite (9) or a highly specialized and unique antipredator trait (8, 10). Here, we describe one of the world's largest and most widespread aposematic complexes: ultrasonic clicking by chemically defended nocturnal moths and their purported mimics.

Moths fly in a dim, nocturnal world, where auditory input can potentially provide substantial information. Over millions of years, they have repeatedly evolved ears (11), organs that likely originated for general auditory surveillance of the environment (12) and that were secondarily co-opted to detect the sonar cries of bats. Hearing organs are found in many regions of the lepidopteran body and occur in a significant majority of species in the order (including ∼85% of species in the megadiverse Macroheterocera) (13–15). These advance-warning sensors allow moths to hear echolocating bats and either evade attack by steering away or performing acrobatic loops, spirals, and dives (16) or respond to bats with a countervailing signal of their own. Ultrasonic clicking by moths, in response to bat sonar, has been documented in tiger moths (17), hawkmoths (18, 19), and one geometrid moth (20). These sounds can function to jam bat sonar (18, 21, 22), signal noxiousness (or mimic noxious acoustic models) (8, 23), and startle bat predators (24).

We hypothesized that, given the efficacy of anti-bat ultrasound production by moths in the hawkmoth and tiger moth lineages, sound emission was perhaps common and widespread across the entire order of more than 160,000 described lepidopteran species. Here, we report a long-term dataset from research across the globe, assaying moth response to playback of bat attack. We tested 252 genera, spanning most families of relatively large-bodied moths (i.e., exceeding 1 cm in length and/or wingspan), and describe anti-bat sound production in 52 genera (21%). For most of these genera, this is behavior never before described. This number is a clear underestimate of acoustic

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The authors declare no competing interest.

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[1]To whom correspondence may be addressed. Email: jessebarber@boisestate.edu or kawahara@flmnh.ufl.edu.

[2]Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

[3]Present address: Environmental Science and Policy, Advanced Academic Programs, Johns Hopkins University, Washington, DC 20036.

[4]Present address: Center for Tropical Research, Institute of the Environment and Sustainability, University of California, Los Angeles, CA 90095.

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aposematism, mimicry, and sonar jamming across this megadiverse insect order [1 in 10 described animals on Earth is a lepidopteran (25)].

Results and Discussion

To uncover the prevalence of ultrasonic response to echolocating bat attack, we trapped moths with ultraviolet (UV) lights and broadcast prerecorded bat sonar attack sequences to moths in tethered flight, across the world's tropics from Asia and Africa (Malaysian Borneo and Mozambique) to South America (Ecuador and French Guiana). Using an ultrasonic speaker, we played representative calls from species of both frequency-modulated (FM; characterized by short-duration, frequency-sweeping pulses) and constant-frequency [CF; characterized by tonal, long-duration pulses (26)] bats (*SI Appendix*, Fig. S1). We recorded moth responses to playback of sonar attack and found that 52 of 252 tested genera respond acoustically to both types of bat sonar (Fig. 1, Dataset S1, and *SI Appendix*, Supplementary Archive 10)—discoveries that now add nine subfamilies to those known to employ this defense (19, 27, 28). While anti-bat ultrasound has been described and well-studied in arctiines (tiger moths) (28–30) and sphingids (hawkmoths) (18, 19, 31), here, we report that this striking antipredator behavior is widespread across the tapestry of lepidopteran diversity (Fig. 2). In fact, if we extrapolate from our sample, ∼20% of the estimated 100,000 species of Macroheterocera (12) produce ultrasound in response to bat sonar.

In addition to playback of bat attack, we also queried moths for ultrasonic response to handling. We simulated a physical predatory attack by grasping the thorax, abdomen, and head. Nearly all moth species that broadcast anti-bat sounds upon

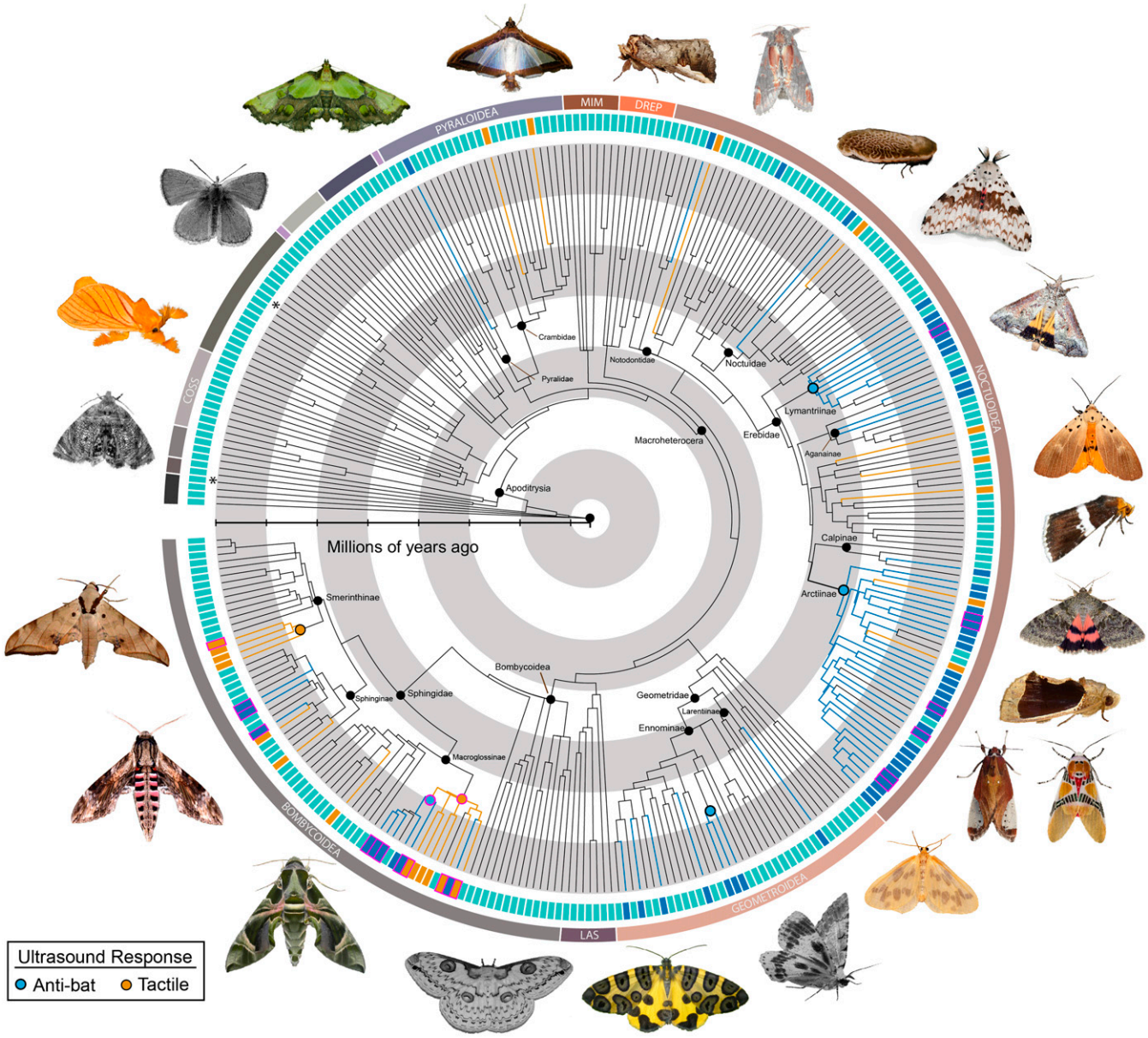

Fig. 1. A molecular phylogeny of Lepidoptera indicating antipredator ultrasound production across the order. Bars and nodes with magenta outlines represent taxa associated with sufficiently large duty-cycle values (>18%) for sonar jamming. Asterisks indicate taxa known to produce ultrasound, but not in response to either tactile stimuli or bat ultrasound. Grayscale images indicate taxa that do not produce ultrasound. This phylogeny is meant to illustrate the diversity of ultrasound production and offer broad strokes on the origins of antipredator sounds at the family and subfamily level, not as a test of evolutionary relationships. Photographs are distributed under Creative Commons Attribution NonCommercial Licenses (see *SI Appendix*, Fig. S2 and Dataset S3 for full accreditations).

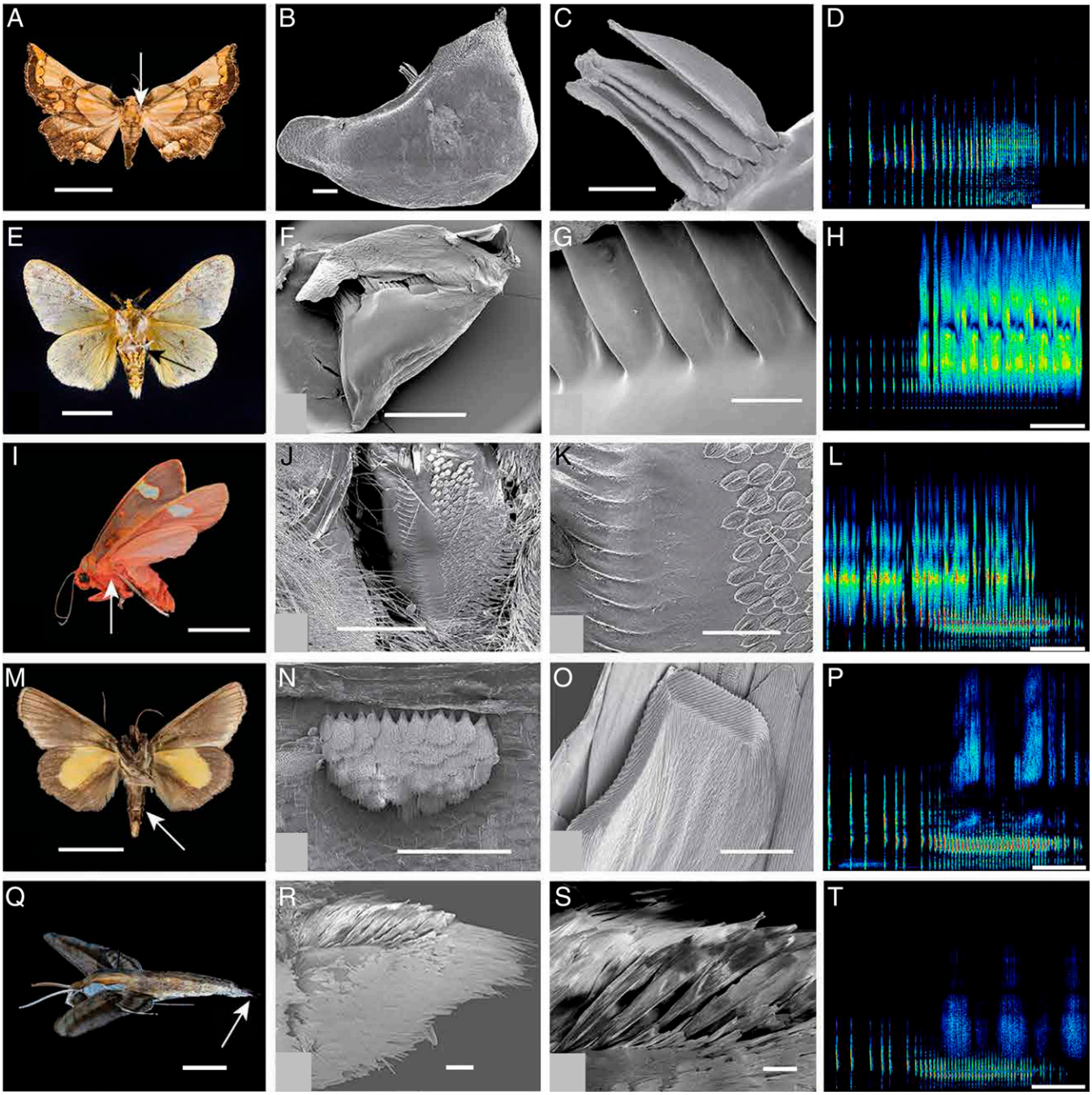

Fig. 2. Anti-bat ultrasound-producing structures. (*A–D*) *M. hampsoni* (Pyralidae: Pyralinae) produces ultrasonic clicks in flight via modified scales on the tegula. (Scale bars, 1.0 cm [*A*]; tegula, 0.2 mm [*B*]; tegular scales, 50 μm [*C*]; response to bat sonar playback [*M. hampsoni*], 100 ms [*D*].) (*E–H*) *Lymantria sp.* (Erebidae: Lymantriinae) generates ultrasound with paired tymbals recessed in abdominal pockets. (Scale bars, 1.0 cm [*E*]; one tymbal represented, 0.5 mm [*F*]; close-up of tymbal, 0.03 mm [*G*]; response to bat sonar playback [*Lymantria sp.*], 100 ms [*H*].) (*I–L*) *Melese sp.* (Erebidae: Arctiinae) emits ultrasound with paired thoracic tymbals. (Scale bars, 1.0 cm [*I*]; tymbal 0.5 mm [*J*]; close-up of microstriations on tymbal surface, 0.1 mm [*K*]; response to bat sonar playback [*Melese peruviana*], 100 ms [*L*].) (*M–P*) *Gonodonta sicheas* (Erebidae: Calpinae) produces ultrasound by stridulating modified abdominal scales. (Scale bars, 1.0 cm [*M*]; patch of stridulatory scales, 0.5 mm [*N*]; stridulatory scale, 50 μm [*O*]; response to bat sonar playback [*Gonodonta bidens*], 100 ms [*P*].) (*Q–T*) *Xylophanes falco* (Sphingidae: Macroglossinae) produces ultrasound by stridulating modified genital valves. (Scale bars, 1 cm [*Q*]; patch of stridulatory scales on genital valve, 0.5 mm [*R*]; stridulatory scales, 0.2 mm [*S*]; response to bat sonar playback [*Xylophanes amadis*], 100 ms [*T*].)

hearing sonar also produced ultrasonic disturbance sounds when handled. Three subfamilies from three different families (Erebidae: Erebinae, Crambidae: Spilomelinae, and Sphingidae: Smerinthinae; Dataset S2) produced ultrasound only in response to tactile stimulation. Producing ultrasound in response to touch may be a generalized antipredator response intended to startle attackers (32). Moreover, responding to bats during handling may still provide time for bats to recognize the warning signal and drop these moths unharmed (27), as bats often first contact their prey with an outstretched wing, directing the insect to their tail membrane, and then subsequently to their mouth (33). Indeed, in a study that pit northern long-eared bats (*Myotis septentrionalis*) against aposematically clicking dogbane tiger moths (*Cycnia tenera*), 75% of signaling moths that were captured were subsequently dropped unscathed (34). The critical experiments pitting bats against moths that produce ultrasound in response to physical contact only have yet to be performed.

Our data indicate that ultrasound production has arisen repeatedly in novel and convergent forms. To determine the mechanism of ultrasonic clicking in each discovered sound producer, we recorded synchronized audio and macro medium-speed video (∼100 frames per second) footage of moths producing ultrasound (Movies S1 and S2). We found several different mechanisms across and within lineages and a great deal of morphological convergence (Fig. 2). The sound-producing mechanisms we uncovered can be grouped into three broad categories: 1) abdominal stridulation, where modified scales on adjoining areas of the moth form a file-scraper device (e.g., Sphingidae: Macroglossinae, Sphingidae: Sphinginae, and Erebidae: Calpinae); 2) percussive wing beating, where sound is produced on each wing stroke by moving the tegula into a striking position between the beating wings (e.g., Pyralidae: Pyralinae); and 3) tymbals, where thin, striated cuticular plates buckle under muscular force and passively release, making a series of clicks during each action due to striations on the tymbal's surface (e.g. , Erebidae: Lymantriinae, Erebidae: Aganainae, and Erebidae: Arctiinae).

Previous work has shown that tiger moths (Erebidae: Arctiinae) and hawkmoths (Sphingidae) use tymbals and stridulation, respectively, to produce ultrasound in response to echolocating bat attack (18, 21, 27). Here, we describe three mechanisms of ultrasound production (Fig. 2): one stridulation-based, one tegula-based, and one tymbal-based. Calpines (a subfamily within Erebidae, here represented by the genus *Gonodonta*) stridulate by using modified ventral abdominal scales (Fig. 2 *M*–*P* and Movie S1) that produce remarkably similar sounds to sphingids, which stridulate with modified scales on the genital valves (refs. 18 and 19 and Fig. 2 *Q*–*T*). We found the percussive wing-beating strategy in only one pyralid moth, *Mittonia hampsoni*, that facultatively beats its wings against its tegula (a structure that plays a role in protecting the base of the forewing; Fig. 2 *A*–*D*) in flight, which we confirmed via ablation experiments. Lymantriines (Erebidae) use paired abdominal tymbals hidden within pockets that form horn-like structures when opened (Fig. 2 *E*–*H* and Movie S2), beaming ultrasound backward at attacking bats.

Aganaines (Erebidae) use paired metathoracic tymbals in the identical positions to arctiines, calling into question the tymbal as a uniting characteristic of arctiines (tiger moths) (35, 36). Previous work described a geometrid (Geometridae: Larentiinae) that uses prothoracic tymbals to generate ultrasonic warning sounds (20). Here, we discovered that multiple genera in a different geometrid subfamily, Ennominae, also produce anti-bat emissions. We have been unable to find a prothoracic tymbal in this group, presenting the intriguing possibility that anti-bat sound production has originated independently at least twice in geometrids. Despite our efforts in the field and museum, there are several other moth subfamilies in which we have confirmed ultrasound production for which we do not know the underlying mechanism (Crambidae: Spilomelinae, Erebidae: Erebinae, Erebidae: Hypocalinae, Noctuidae: Hadeninae, Noctuidae: Noctuinae, Notodontidae: Notodontinae, and Notodontidae: Nystaleinae). Clearly, the mechanisms driving the acoustic arms race between moths and bats are myriad and diverse.

We also discovered an interesting form of ultrasound production in the Dalceridae (genus *Acraga*). These noneared animals constantly produce ultrasound while in flight, similar to the behaviors previously described in other small-bodied non-Macroheterocera (37, 38). The mechanism of sound production in the *Acraga* genus remains unknown—the wing-based aeroelastic tymbals implicated in sound production in other non-Macroheterocera do not appear responsible. Considering that moths in the genus *Acraga* are unpalatable to bats (*SI Appendix*), it is tempting to assert that these sounds are involved in advertising noxious taste to echolocating bats. Until moths using this type of ultrasound production are pitted against bats in appropriate experiments, the function of these sounds will remain unclear.

To better understand how the interactions between bats and sound-producing moths might play out across the night skies, we quantified moth acoustic emissions, using previously described parameters to capture the temporal and spectral components (27). We found that animals that produce ultrasound to playback of bat attack emit frequencies centered around ∼65 kHz [± ∼40 to 110 kHz at 15-dB range; matching the frequency of best hearing in most bat species (39, 40)] and a substantial range of duty cycles (sound per unit time; *SI Appendix*, Supplementary Archive S10). While it is possible that a sound of any duty cycle can startle naive bats or warn of noxious taste (or mimic chemically protected models), only acoustic emissions with high-duty cycles can jam bat sonar (8, 10, 18, 22, 41, 42). In fact, duty cycles of at least 18% (this parameter is sensitive to analysis approaches) seem to be necessary to interfere with the processing of returning echoes from echolocating bats (18). Importantly, this threshold is necessarily derived from a limited number of bat–moth interaction studies, and we predict that jamming efficacy amplifies as duty cycle increases. In our dataset, we find preliminary evidence of independent origins of sonar jamming in at least six moth subfamilies (Sphinginae, Macroglossinae, Aganainae, Arctiinae, Calpinae, and Lymantriinae) based on this threshold. A seventh subfamily (Smerinthinae) also independently developed duty cycles theoretically sufficient to jam sonar, yet they are not capable of this behavior, as this group lacks ears and thus cannot respond in advance to attacking bats. Animals that use complex tymbals with multiple microstriations (aganines, arctiines, and lymantrids) and stridulatory mechanisms (calpines and sphingids) are also likely capable of jamming. Thus, although moth morphology is not strictly deterministic of sound-production function, some morphologies [wing-beating mechanisms and tymbals with few microstriations (43)] cannot support the high-duty cycle (and likely high intensity) sounds necessary for jamming (18, 22).

Sonar jamming appears to be a derived strategy that has arisen repeatedly and recently in multiple lineages. Our preliminary investigations indicate that this strategy is not uniformly related to a loss (or lack of gain) of unpalatability to bats. We find that some genera capable of jamming bat sonar are palatable (Dataset S2; see *Methods* for palatability experimental details) and other genera are not, sometimes within the same subfamily (Arctiinae and Lymantriinae); thus, the hypothesis that the origin of duty cycles capable of jamming frees lineages from the costs of sequestering chemicals for protection against bats (44) seems unlikely to be commonly supported. One possibility is that host-plant specialization canalizes sequestration strategies. Advertising difficulty of capture (evasive aposematism) is another conceivable function of conspicuous high-duty-cycle sounds (45) that may operate alongside sonar jamming; however, this hypothesis remains untested.

It appears that most sound-producing moths are not capable of jamming bat sonar. The majority of sound producers are therefore likely communicating with their bat predators, rather than disrupting echolocation. We found that moth genera that produced anti-bat sounds were commonly split between those

that were palatable to bats and those that were not. Geometrid moths indeed seemed to be noxious, but not as repellent as lymantrids or arctiines (Dataset S2). Multiple subfamilies (Calpinae, Erebinae, Noctuinae, Nystaleinae, Macroglossinae, Smerinthinae, and Sphinginae) were considered quite palatable by the bats we pit these moths against (*SI Appendix*). These results likely indicate that these animals are exploiting the education imparted to their predators by unpalatable models (i.e., they are Batesian mimics).

To test the possible organization of anti-bat sounds into acoustic mimicry rings, we intensively studied a community of moths in Sumaco, Ecuador. We captured moths with UV lights and queried this megadiverse community for anti-bat acoustic response over 14 continuous nights. To analyze the resulting acoustic data, we used a dimensionality reduction algorithm [UMAP: Uniform Manifold Approximation and Projection (46)] to find groups of moths with similar acoustic features (clusters). This unsupervised machine-learning algorithm estimates the topology of high-dimensional data and uses this information to build a low-dimensional representation that preserves relationships present in the data. We used 10 acoustic features (*Methods*) and 33 species as input to UMAP to project the data from a 10-dimensional space into a two-dimensional (2D) space, where we found five well-separated clusters (Fig. 3; interactive three-dimensional [3D] visualization at: projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json).

While we caution that this analysis offers only a cursory temporal and spatial snapshot of the hyperdiverse mimetic associations that are likely present, we find some remarkable patterns. Each cluster of moth anti-bat sounds includes at least one genus that we have found to be unpalatable to bats, and most clusters also contain animals that bats readily consume. For example, one acoustic cluster contains one unpalatable dalcerid (Dalceridae), five palatable calpines (Erebidae: Calpinae), and two palatable sphingids (Sphingidae: Macroglossinae). Another cluster consists of six geometrid species (Ennominae) and one tiger moth (Erebidae: Arctiinae), all of which are likely honestly advertising noxious taste—perhaps a Müllerian ensemble. Interestingly, one cluster of Arctiini tiger moths (Erebidae: Arctiinae) uniformly contains extremely high-duty-cycle genera capable of jamming bat sonar, including two genera that appear to be unpalatable to bats, supporting the prediction that jamming and aposematism are not mutually exclusive (27). Our preliminary data portend substantial community-level structuring of ultrasonic warning signals driven by the psychologies of syntopic bat predators (47). We are at the frontier of understanding a hidden dimension of biodiversity—the ultrasonic information transfer between bats and their insect prey.

Importantly, many species of moths also use ultrasonic sounds to transmit information to conspecifics—with males from at least six families (Crambidae, Erebidae, Geometridae, Noctuidae, Pyralidae, and Sphingidae) likely using this strategy to attract mates (48, 49). Some male moths use intense ultrasonic signals to communicate with females, as in tiger moths (Erebidae: Arctiinae) (49). Other families of moths produce quiet mating calls (Noctuidae, Arctiidae, Geometridae, and Crambidae), apparently intended for nearby females (49). These "whispering" moths likely employ soft signals to avoid detection by eavesdropping bats and other predators (50–52). It is unclear if the use of ultrasound by moths evolved first in a mating context or if it was secondarily co-opted from an anti-bat origin. Some moths are able to discriminate mates from bats, such as *Achroia grisella* (Pyralidae) females that exhibit differing behaviors, positive phonotaxis or freezing, when stimulated by different pulse rates [a higher pulse rate indicating a conspecific calling male and a lower pulse rate indicating an approaching bat, respectively (53)]. Alternatively, female *Spodoptera litura* (Noctuidae) are unable to distinguish attacking bats from ultrasound-producing males, suggesting a sensory-exploitation origin of sound production in moths—that is, male moths exploit female freezing behavior to secure matings (54). We do not yet know whether moths that acoustically respond to echolocating bats are more likely to use ultrasound for mating, as many moths have not yet been tested for these behaviors (55), but this notion seems likely.

Ultrasonically signaling moths appear to be connected by some of the most widespread and biodiverse mimicry complexes

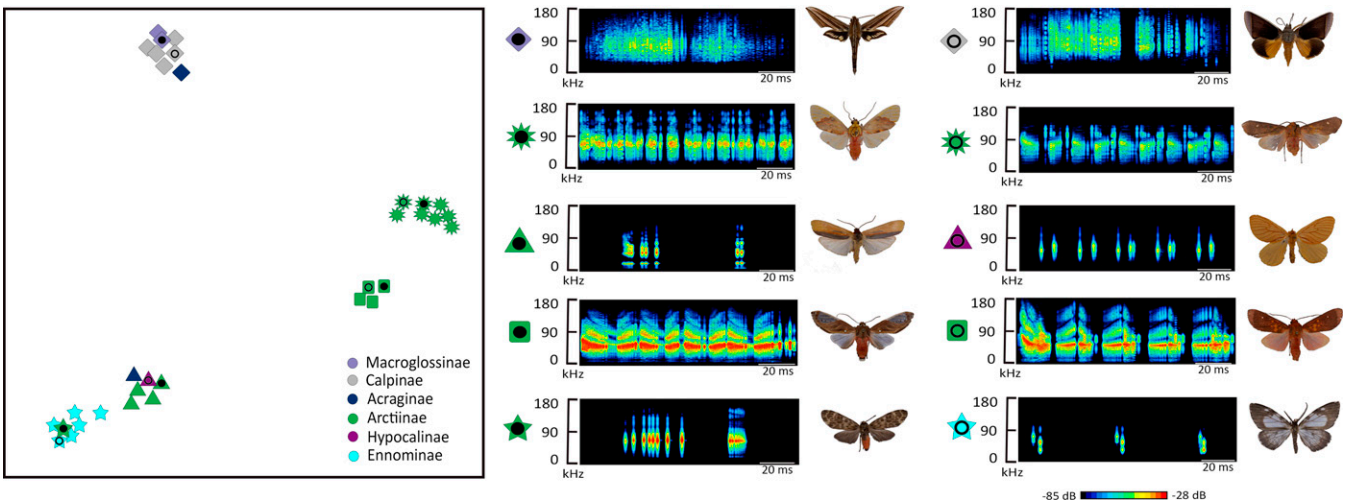

Fig. 3. Purported acoustic mimicry rings of a community of moths in Sumaco, Ecuador (33 species). A UMAP projection shows clusters of moth anti-bat sounds with similar acoustic features. The relative distance between the clusters is meaningful in the sense that clusters that are close in the 2D map are more similar than clusters that are further away. Photos of moths are congeners at the genus level. All photos were taken by the authors. *Xylophanes titana,* purple diamond, solid circle; *Gonodonta syrna*, gray diamond, open circle; *Scaptius ditissima*, green sun, solid circle; *Melese sordida*, green sun, open circle; *Agylla sp*., green triangle, solid circle; *Acraga moorei*, dark-blue triangle, open circle; *Bertholdia bilineola*, green square, solid circle; *Melese chozeba*, green square, open circle; *Eucereon formosum dognini*, green star, solid circle; *Nephodia* sp., blue star, open circle. See *SI Appendix*, Supplementary Archive 11 for available palatability data.

known to date (56, 57). The dynamics of these associations stand as a great unknown in natural history and a laboratory for understanding mimicry dynamics and convergent evolution (58). The intense pressure to thwart the attacks of echolocating bats seems to have also driven ultrasound production in other insects. Tiger beetles (Cicindelidae) produce ultrasonic warning signals in response to sonar playback (59), and some fireflies (Lampyridae: Lampyrinae), known to be noxious to bats (60), constantly produce ultrasonic clicks in flight, which may serve as a component of a multimodal aposematic signal to bats (61). We predict that a complete understanding of ultrasonic mimicry rings will involve a thorough analysis of all major nocturnal, aerial insect groups, including moths (Lepidoptera), beetles (Coleoptera), true bugs (Hemiptera), flies (Diptera), lacewings and antlions (Neuroptera), and more. Understanding how bat receivers generalize the massive numbers of insect warning sounds into categories is an important frontier in understanding this powerful selective force. Bats have shaped the nocturnal soundscape in profound ways—driving a chorus of nightly cries across the globe, as moths and perhaps other insects jam sonar, warn of noxious chemicals, and mimic the sounds of unpalatable models. Comprehending this symphony is central to understanding insect biodiversity.

Methods

Statement on Fieldwork Ethics. During our data-collection trips, we received assistance, guidance, and hospitality from people in each of our field sites, whose names we did not document. We recognize that this kind of expedition science is problematic and can be harmful to these communities in a variety of ways, including perpetuating colonial practices. In the future, we will strive to engage more deeply with the local population in the areas where we work and to offer more educational and professional opportunities. We remain indebted to those who helped us along this multiyear journey.

Echolocation Playback, Tactile Stimulation, and Acoustic Recording. We assayed moths in three of the world's tropics: South America (Ecuador and French Guiana), Africa (Mozambique), and Asia (Malaysian Borneo) for ultrasonic reply to handling and bat attack. To simulate handling by a predator, we lightly compressed the moth's head, abdomen, or thorax. To query moths for response to playback of bat echolocation attack, we tethered moths in flight after dark to elicit the most natural anti-bat behaviors. We used various sizes of locking forceps to tether most moths, making sure we were not inhibiting acoustic response by our tethering technique. We tethered large moths (e.g., sphingids and saturniids) using a "lasso" technique, where we tied fishing line between the abdomen and thorax and fed the line through a hollow plastic rod that was secured in forceps. We simulated bat attack using six recorded bat echolocation attack sequences (see *SI Appendix*, Fig. S1 for spectrograms). Bat assemblages and echolocation strategies vary across the world. To capture some of the diversity of echolocation calls that moths might experience in different tropical regions, we presented moths with three different FM echolocation attacks and two CF attacks. The echolocation sequences we used were from bat species with distributions that often did not overlap the moths we tested. However, as many of the characteristics of CF and FM sonar strategies are conserved across bat species, the files we presented were biologically relevant. Two of the FM sequences were recorded from individual trained bats attacking a moth tethered 10 cm from a microphone (FM1, *Lasiurus borealis*; FM2, *Eptesicus fuscus*) (19), allowing us to present echolocation calls to tethered moths that closely matched the pattern of received sound level that a moth would experience during a sonar-driven attack in the wild. We also generated a synthetic bat attack based on the short-duration, broadband echolocation cries of some bats. Our aim was to create a sonar attack that did not include buzz II, as found in many bat families, including Phyllostominae, Craseonycteridae, and Molossidae. We followed the pulse presentation rate of *Macrophyllum macrophyllum* (62) (synthetic). To represent CF bat calls, we used on-board telemike recordings of two individual bats (*Rhinolophus ferrumequinum nippon*) attacking prey provided to us by Yuki Kinoshita and Shizuko Hiryu, Doshisha University, Kyoto, Japan (63) (CF1 and CF2). The on-board recording of these calls again allowed us to closely match the sound level that a moth would experience during a bat attack in the wild. We presented this suite of sonar attacks in a randomized order with at least 1 s between playbacks and made sure that the moth was in steady flight and silent (no ultrasound production) before each echolocation attack was broadcast. All bat calls were played through an Avisoft UltraSoundGate Player BL Pro Speaker/Amplifier (±6 dB, 20 to 110 kHz, playback sampling rate 250 kHz) placed 10 cm behind the moth's abdomen, except in the cases of sphingid moths, where the speaker was positioned on-axis 10 cm from the moth's face, as their mouthparts comprise their hearing organs (64). Similarly, we recorded moth sounds using an Avisoft CM16 condenser microphone (±3 dB, 20 to 140 kHz) attached to an UltraSoundGate 116Hme data acquisition device sampling at 375 kHz via a laptop computer running Avisoft Recorder software, placed at a 90° angle 10 cm from the moth's thorax, except in the cases of sphingid moths, where the microphone was placed 10 cm directly behind the moth [as the genitals were previously known as the sound-producing organs in this group (19)].

Regardless of mechanism of ultrasound production, we focused our analyses on one complete modulation cycle of sound, which we defined as the two-component structure of the sound emissions. This paired structure results from: 1) the up–down wing stroke, 2) the buckling–unbuckling of tymbals, or 3) the in–out or side–side stridulating of valves. We used Avisoft SASLab Pro software to measure three modulation cycles from each individual in our dataset, except in cases where only two could be measured. We extracted the same parameters as those described in Barber and Conner (27) for comparability to other studies. To measure the temporal characteristics–duty cycle (proportion of 100-ms window with moth sound present), duration of modulation cycle, and duration of modulation-cycle components–we used the pulse-train analysis tool with the following settings (time constant = 0.025 ms, threshold = 0.15 V, hysteresis = 15 dB, start/end threshold = −15 dB, envelope = rectification + exponential decay, and pulse detection = peak search with hysteresis). We measured spectral characteristics–dominant frequency, frequency 15 dB above and below dominant frequency–from the Power Spectrum (averaged) tool with a Hann evaluation window and fast Fourier transform = 1,024.

For downstream analyses, we only considered a species to be responsive (i.e., producing ultrasound in response to bat ultrasound and/or tactile stimuli) if we recorded responsive ultrasound production in at least two specimens. Otherwise, the recorded species were assumed to be nonresponsive. This is not the preferred method for obtaining negative data, since it is plausible that a moth could be capable of responding to stimuli, yet did not do so in our setting. Thus, moths that we tested and found to be nonresponsive in the field are included in our phylogenetic analyses as negative data, whereas moths that are present in our phylogenetic tree, but that we did not test in the field, are included in our analyses as missing data (*Phylogenetic Methods*).

Palatability. Palatability experiments were conducted on 93 moths from 26 species (*SI Appendix*) in the field. We ablated sound-producing structures (if present), before offering a hand-held captive bat (see *SI Appendix* for species and locations) a moth via forceps. In an attempt to control for the foraging motivation of each bat, we only scored interactions where the bat was willing to eat a control moth (a species we knew to be palatable) both before and after we offered an experimental moth. We scored partial palatability by dividing the length of the moth body into six parts and assigning one point to the head, two points to the thorax, and three points to the abdomen, following the methods of Hristov and Conner (41). A palatability score of 0 indicates the moths was entirely rejected, and a score of 6 indicates the moth was 100% consumed.

Unsupervised Machine-Learning Cluster Analysis of Moth Sounds. The dimensionality-reduction algorithm UMAP (46) was used for finding groups of moth sounds with similar features (clusters). Dimensionality-reduction algorithms capture variability in a limited number of random variables to allow 2D or 3D visualization of data that reside in a multidimensional space. The most common approach is the method of principal component analysis (PCA) (65), which uses linear combinations of variables to generate orthogonal axes that capture the variation present in the data with fewer variables. Another approach, developed a century after PCA, *t*-Distributed Stochastic Neighbor Embedding (t-SNE) (66), carries out dimensionality reduction by analyzing the similarity of

points using a Gaussian distance in high-dimensional space and mapping these data into a low-dimensional space. t-SNE is able to capture local nonlinear relationships in the data, which PCA by its linear design is not able to, but does not capture the global structure. A more recent method, UMAP, is an unsupervised machine-learning algorithm for dimension reduction based on manifold learning techniques and ideas from topological data analysis. It works by estimating the topology of the high-dimensional data and uses this information to build a low-dimensional representation that preserves relationships present in the data. It is better at mapping the global structure of the data from the high-dimensional space than t-SNE and is able to capture local relationships as well.

We used the moth acoustic features to define a multidimensional space where each moth is represented by a vector (or point) in that space. The dataset consisted of 33 entries with 10 features each, which translates to 33 points (vectors) in a 10-dimensional space. We input their coordinates into a PCA as a preprocessing step. The resultant principal components were then used as input to UMAP to project the data from the 10-dimensional space into a 2D space. Each cluster shares similar features. The relative distance between the clusters is meaningful in the sense that clusters that are close in the 2D map are more "similar" than clusters that are farther away. The features variables used, extracted from audio files, were "MC DC mean," "d MC mean," "D 1/2 mean," "D silent mean," "D 2h mean," "DF mean," "D dB mean," "+ 15 dB mean," "−15 dB mean," and "100 ms DC mean" (see *SI Appendix* for definitions). We used the software tools Scikit-learn (67) and pandas (68). The steps of dimensional reduction using the different methods we have discussed above can be seen in the interactive online version of the embedding (projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json) by clicking on the different bookmarks on the right (created via ref. 69).

Phylogenetic Methods. In order to determine the timing of evolution of anti-bat sound production in Lepidoptera, we created a dated molecular phylogeny, using the ages estimated in the Lepidoptera phylogeny of Kawahara et al. (12), that incorporates the moth taxa we tested for anti-bat ultrasound production. We attempted to find previously published cytochrome c oxidase subunit I (COI) barcodes and five commonly sequenced nuclear genes (CAD, DDC, EF1-A, period, and wingless) for one species of every genus that was tested for anti-bat sound production [as well as the sound-producing genus tested in Corcoran and Hristov (20)] and also used published data from as many species as possible that were included in the Kawahara et al. (12) dataset (this transcriptomic dataset lacked data for these six genes and thus could not directly be used). Whenever possible, molecular data for a genus were represented by a tested species; when such data were not available (after searching both the National Center for Biotechnology Information (NCBI) and Bold Taxonomy Browser), a congener was used instead.

There were 11 genera from our sound-production dataset that had no available sequence data; in order to represent these taxa in our analysis, we obtained new COI barcodes from DNA extracted from the legs of the ensonified specimens. DNA was extracted by using an OmniPrep Genomic DNA Extraction Kit (G-Biosciences), following the protocol of Espeland et al. (70), and PCR was performed following the protocol of Hebert et al. (71), using Lep1 reverse primers. Sanger sequencing was performed by Genewiz. COI sequencing was unsuccessful for two non-sound-producing genera (*Grammodora* and *Trotonotus*), which were consequently excluded from the analysis. The nine sequenced barcodes used in this analysis were uploaded to NCBI (all accession nos. provided in Dataset S4), and specimen vouchers were deposited at the McGuire Center for Lepidoptera and Biodiversity (Dataset S4). In total, our molecular dataset contained at least one gene for 432 Lepidoptera species.

Sequences for the six genes were aligned in MAFFT (72), then manually trimmed and concatenated in GENEIOUS version (v).11.1.5. The dataset was partitioned by codon position, constrained using the topology in figure 1 of Kawahara et al. (12), and a maximum-likelihood analysis was performed in IQ-TREE v.1.6.2 (73), using ModelFinder to determine the best-fit substitution models for each partition (74). The resulting maximum-likelihood tree was dated in TreePL (75), using the age estimates from Kawahara et al. (12) as secondary calibrations. The molecular dataset and other files associated with these analyses are included in *SI Appendix*, Supplementary Archives 1–9.

Two ancestral state reconstructions (ASRs) of anti-bat sound production were performed by using stochastic character mapping with the "make.simmap" in the R package Phytools v07-70 (76). Symmetrical transition-rate models were used in both ASRs, and 1,000 simulations were performed. In order to reduce the amount of computational resources required, these ASRs were performed only on the Ditrysia clade of the dated tree, which comprise 93% of all taxa in the analysis (400/432). Only one non-Ditrysian genus had been tested for ultrasound production (Hepialidae: *Dalaca*, which did not produce ultrasound), so their absence did not significantly impact the ASR results since only 1/32 could have been confidently assigned a character state. In the first ASR, the evolution of anti-bat sound production was assessed by treating it as a ternary character, with taxa assigned to one of the following: 1) no sound production in response to a stimulus (this includes genera that constantly produce sound, regardless of whether there is a stimulus; e.g., *Acraga*); 2) sound production in response to tactile stimuli; or 3) sound production in response to both tactile stimuli and bat ultrasound (Dataset S5 and *SI Appendix*, Fig. S3). In instances where a species in the ensonified dataset was represented in the molecular dataset by a congener, we assumed that the congener had an identical character state. For taxa in the Kawahara et al. (12) dataset that were included in our maximum-likelihood analysis, but not ensonified, an equal probability of 1/3 was assigned to each of the three states, if those taxa were known to have ears. For the untested taxa known to lack ears (12), we assumed that they could not detect ultrasound and thus had no way to respond to bat calls, and we consequently assigned equal probabilities of 1/2 to the first two states and 0 to the third state.

In the second ASR, the evolution of anti-bat sound production capable of jamming bat sonar [i.e., anti-bat ultrasound with a duty cycle value of at least 18% (18)] was assessed by treating it as a binary character. Taxa were assigned to one of the following: 1) duty cycle less than 18% (this includes genera that did not produce any ultrasound when tested); or 2) duty cycle of 18% or greater (Dataset S6 and *SI Appendix*, Fig. S4). As with the previous ASR, we assumed that congeners had identical character states. If duty-cycle data were collected for multiple species in a genus, the value from the species with the largest mean duty cycle was used for that genus in the ASR (*SI Appendix*, Supplementary Archive 10). For untested taxa in the Kawahara et al. (12) dataset that were included in our maximum-likelihood analysis, but not ensonified, an equal probability of 1/2 was assigned to each of the two states (regardless of whether they had ears). We also performed an ASR using maximum likelihood ["anc.ML" in Phytools v07-70 (76)] that modeled duty cycle as a continuous character (Dataset S7 and *SI Appendix*, Fig. S5). However, since this method cannot incorporate taxa with missing data, all nonensonified taxa were assumed to have duty cycles of 0%.

Data and Materials Availability. The sequenced DNA barcodes used in this study have been deposited in the NCBI GenBank sequence database (accession nos.: ON116351–ON116359, and are also provided in Dataset S4) (77, 78). All other data are available in the main text, the supporting information, or at the Dryad Digital Repository (https://doi.org/10.5061/dryad.2v6wwpzqt) (79).

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Author affiliations: [a]Department of Biological Sciences, Boise State University, Boise, ID 83725; [b]McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL 32611; [c]Department of Biology, University of Florida, Gainesville, FL 32611; [d]Precision Genomics, Intermountain Healthcare, St. George, UT 84790; [e]Entomology Laboratory, Museo de Zoología, Universidad del Azuay, Cuenca, 010107, Ecuador; [f]Consejo Nacional de Investigaciones Científicas y Técnicas, Patagonia Norte, Ingeniero White, 8400, Argentina; [g]Departamento Materiales Nucleares, Centro Atómico Bariloche, Comisión Nacional de Energía Atómica, Bariloche, 8400, Argentina; and [h]Instituto de Investigaciones en Biodiversidad y Medioambiente, Universidad Nacional del Comahue, Bariloche, 8400, Argentina

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