

## Anti-Bat Ultrasound in Moths

### 1 **Anti-Bat Ultrasound Production in Moths is Globally and Phylogenetically** 2 **Widespread**

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4 Barber JR<sup>1\*</sup>, Plotkin D<sup>2</sup>, Rubin JJ<sup>2,3</sup>, Homziak NT<sup>2</sup>, Leavell BC<sup>1^</sup>, Houlihan P<sup>2,3,#</sup>, Miner  
5 KA<sup>1</sup>, Breinholt JW<sup>4</sup>, Quirk-Royal B<sup>1</sup>, Padrón PS<sup>5,2</sup>, Nunez M<sup>6,7,8</sup>, Kawahara AY<sup>\*2</sup>

6  
7 <sup>1</sup> Department of Biological Sciences, Boise State University, Boise, ID, USA

8 <sup>2</sup> McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History,  
9 University of Florida, Gainesville, FL, USA

10 <sup>3</sup> Department of Biology, University of Florida, Gainesville, FL, USA

11 <sup>4</sup> Division of Bioinformatics, Intermountain Healthcare, Precision Genomics, St. George,  
12 UT, USA

13 <sup>5</sup> Entomology Laboratory, Museo de Zoología, Universidad del Azuay, Cuenca, Ecuador

14 <sup>6</sup> Consejo Nacional de Investigaciones Cientificas y Tecnicas(CONICET), Patagonia  
15 Norte, Argentina

16 <sup>7</sup> Departamento Materiales Nucleares, Centro Atómico Bariloche, Comisión Nacional de  
17 Energía Atómica, Bariloche, Argentina

18 <sup>8</sup> INIBIOMA, Universidad Nacional del Comahue, Bariloche, Argentina

19  
20 <sup>^</sup>current affiliation: Department of Biological Sciences, Purdue University, West  
21 Lafayette, IN, USA

22 <sup>#</sup>current affiliations: Environmental Science & Policy, Advanced Academic Programs,  
23 Johns Hopkins University, Washington, D.C., USA and Center for Tropical Research,  
24 Institute of the Environment & Sustainability, University of California Los Angeles  
25 (UCLA), Los Angeles, CA, USA

26  
27 \*corresponding authors: Jesse R. Barber ([jessebarber@boisestate.edu](mailto:jessebarber@boisestate.edu)) and Akito Y.  
28 Kawahara ([kawahara@flmnh.ufl.edu](mailto:kawahara@flmnh.ufl.edu))

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### 35 **Abstract**

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

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### 53 **Introduction**

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

65       Moths fly in a dim, acoustic world. Over millions of years they have repeatedly  
66 evolved ears (11), organs that likely originated for general auditory surveillance of the  
67 environment (12), and that were secondarily co-opted to detect the sonar cries of bats.  
68 Hearing organs are found in many regions of the lepidopteran body and occur in a  
69 significant majority of species in the order (including ~85% of species in the megadiverse  
70 Macroheterocera) (13–15). These advance warning sensors allow moths to hear  
71 echolocating bats and either motorically evade attack by steering away or performing  
72 acrobatic loops, spirals and dives (16), or respond to bats with a countervailing signal of  
73 their own. Ultrasonic clicking by moths, in response to bat sonar, has been documented in  
74 tiger moths (17), hawkmoths ((18, 19), and one geometrid moth (20). These sounds can

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75 function non-mutually-exclusively to jam bat sonar (18, 21, 22), signal noxiousness (or  
76 mimic noxious acoustic models) (8, 23), and startle bat predators (24).

77 We hypothesized that, given the efficacy of anti-bat ultrasound production by  
78 moths in the hawkmoth and tiger moth lineages, sound emission was perhaps common  
79 and widespread across the entire order of more than 160,000 described lepidopteran  
80 species. Here, we report a long-term dataset from research across the globe, assaying  
81 moth response to playback of bat attack. We tested 252 genera, spanning most families of  
82 relatively large-bodied moths (i.e., exceeding 1 cm in length and/or wingspan), and  
83 describe anti-bat sound production in 52 genera (21%). For most of these genera, this is  
84 novel behavior never before described. This number is a clear underestimate of acoustic  
85 aposematism, mimicry, and sonar jamming across this megadiverse insect order (1 in 10  
86 described animals on Earth is a lepidopteran (25)).

## 87 **Results and Discussion**

88 To uncover the prevalence of ultrasonic response to echolocating bat attack, we  
89 trapped moths with UV lights and broadcast pre-recorded bat sonar attack sequences to  
90 moths in tethered flight, across the world's tropics from Asia and Africa (Malaysian  
91 Borneo and Mozambique) to South America (Ecuador, and French Guiana). Using an  
92 ultrasonic speaker, we played representative calls from species of both frequency-  
93 modulated (FM; characterized by short-duration, frequency-sweeping pulses) and  
94 constant-frequency (CF; characterized by tonal, long-duration pulses (26)) bats (see Fig.  
95 S1). We recorded moth responses to playback of sonar attack and found that 52 of 252  
96 tested genera respond acoustically to both types of bat sonar (Fig. 1, Dataset S1, Supp.  
97 Archive 10) – discoveries that now add nine subfamilies to those known to employ this

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98 defense (19, 27, 28). While anti-bat ultrasound has been described and well-studied in  
99 arctiines (tiger moths) (28–30) and sphingids (hawkmoths) (18, 19, 31), here we report  
100 that this striking anti-predator behavior is widespread across the tapestry of lepidopteran  
101 diversity (Fig 2). In fact, if we extrapolate from our sample, ~20% of the estimated  
102 100,000 species of Macroheterocera (12) produce ultrasound in response to bat sonar.

103         In addition to playback of bat attack, we also queried moths for ultrasonic  
104 response to handling. We simulated a physical predatory attack by grasping the thorax,  
105 abdomen, and head. Nearly all moth species that broadcast anti-bat sounds upon hearing  
106 sonar also produced ultrasonic disturbance sounds when handled. Three subfamilies from  
107 three different families (Erebidae: Erebininae, Crambidae: Spilomelinae, Sphingidae:  
108 Smerinthinae; see Dataset S2) produced ultrasound only in response to tactile stimulation.  
109 Producing ultrasound to touch may be a generalized anti-predator response intended to  
110 startle attackers (32). Moreover, responding to bats during handling may still provide  
111 time for bats to recognize the warning signal and drop these moths unharmed (*sensu*  
112 (27)), as bats often first contact their prey with an outstretched wing, directing the insect  
113 to their tail membrane, and then subsequently to their mouth (33). Indeed, in a study that  
114 pit northern long-eared bats (*Myotis septentrionalis*) against aposematically clicking  
115 dogbane tiger moths (*Cycnia tenera*), 75% of signaling moths that were captured were  
116 subsequently dropped unscathed (34). The critical experiments pitting bats against moths  
117 that produce ultrasound to physical contact only have yet to be performed.

118         Our data indicate that ultrasound production has arisen repeatedly in novel and  
119 convergent forms. To determine the mechanism of ultrasonic clicking in each newly  
120 discovered sound producer, we recorded synchronized audio and macro medium-speed

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121 video (~100 fps) footage of moths producing ultrasound (see Movies S1–S2). We found  
122 several different mechanisms across and within lineages, and a great deal of  
123 morphological convergence (Fig. 2). The sound-producing mechanisms we uncovered  
124 can be grouped into three broad categories: 1) abdominal stridulation, where modified  
125 scales on adjoining areas of the moth form a file-scraper device (e.g., Sphingidae:  
126 Macroglossinae, Sphingidae: Sphinginae, Erebidae: Calpinae); 2) percussive wing  
127 beating, where sound is produced on each wing stroke by moving the tegula into a  
128 striking position between the beating wings (e.g., Pyralidae: Pyralinae); and 3) tymbals,  
129 where thin, striated cuticular plates buckle under muscular force and passively release  
130 often making a series of clicks during each action due to striations on the tymbal's  
131 surface (e.g., Erebidae: Lymantriinae, Erebidae: Aganainae, Erebidae: Arctiinae).

132       Previous work has shown that tiger moths (Erebidae: Arctiinae) and hawkmoths  
133 (Sphingidae) use tymbals and stridulation, respectively, to produce ultrasound in response  
134 to echolocating bat attack (18, 21, 27). Here we describe three new mechanisms of  
135 ultrasound production (Fig. 2): one stridulation-based, one tegula-based, and one tymbal-  
136 based. Calpines (a subfamily within Erebidae, here represented by the genus *Gonodonta*)  
137 stridulate using modified ventral abdominal scales (see Fig. 2M-P, Movie S1) that  
138 produce remarkably similar sounds to sphingids, which stridulate with modified scales on  
139 the genital valves (18, 19); Fig. 2Q-T). We found the percussive wing beating strategy in  
140 only one pyralid moth, *Mittonia hamponi*, that facultatively beats its wings against its  
141 tegula (a structure that plays a role in protecting the base of the forewing; Fig. 2A-D) in  
142 flight, which we confirmed via ablation experiments. Lymantriines (Erebidae) use paired

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143 abdominal tymbals hidden within pockets that form horn-like structures when opened  
144 (see Fig. 2E-H, Movie S2), beaming ultrasound backwards at attacking bats.

145       Aganaines (Erebidae) use paired metathoracic tymbals in the identical positions to  
146 arctiines, calling into question the tymbal as a uniting characteristic of arctiines (tiger  
147 moths) (35, 36). Previous work described a geometrid (Geometridae: Larentiinae) that  
148 uses prothoracic tymbals to generate ultrasonic warning sounds (37). Here we discovered  
149 that multiple genera in a different geometrid subfamily, Ennominae, also produce anti-bat  
150 emissions. We have been unable to find a prothoracic tymbal in this group, presenting the  
151 intriguing possibility that anti-bat sound production has originated independently at least  
152 twice in geometrids. Despite our efforts in the field and museum, there are several other  
153 moth subfamilies in which we have confirmed ultrasound production for which we do not  
154 know the underlying mechanism (Crambidae: Spilomelinae, Erebidae: Erebininae,  
155 Erebidae: Hypocalinae, Noctuidae: Hadeninae, Noctuidae: Noctuinae, Notodontidae:  
156 Notodontinae, Notodontidae: Nystaleinae). Clearly, the mechanisms driving the acoustic  
157 arms race between moths and bats are myriad and diverse.

158       We also discovered an interesting form of ultrasound production in the Dalceridae  
159 (genus *Acraga*). These non-eared animals constantly produce ultrasound while in flight  
160 similar to the behaviors previously described in other small-bodied non-Macroheterocera  
161 (38, 39). The mechanism of sound production in the *Acraga* genus remains unknown –  
162 the wing-based aeroelastic tymbals implicated in sound production in other non-  
163 Macroheterocera do not appear responsible. Considering that moths in the genus *Acraga*  
164 are unpalatable to bats (see supplement), it is tempting to assert that these sounds are  
165 involved in advertising noxious taste to echolocating bats. Until moths using this type of

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166 ultrasound production are pit against bats in appropriate experiments, the function of  
167 these sounds will remain unclear.

168       To better understand how the interactions between bats and sound-producing  
169 moths might play out across the night skies, we quantified moth acoustic emissions, using  
170 previously-described parameters to capture the temporal and spectral components (27).  
171 We found that animals that produce ultrasound to playback of bat attack emit frequencies  
172 centered around ~65 kHz ( $\pm$  ~40-110 kHz at 15 dB range; matching the frequency of best  
173 hearing in most bat species (40, 41)) and a substantial range of duty cycles (sound per  
174 unit time; see Supp. Archive S10). While it is possible that any duty cycle sound can  
175 startle naive bats, or warn of noxious taste (or mimic chemically-protected models), only  
176 high duty cycle sounds can jam bat sonar (8, 10, 18, 22, 42, 43). In fact, duty cycles of at  
177 least 18% (this value is sensitive to analysis approaches) seem to be necessary to interfere  
178 with the processing of returning echoes from echolocating bats (Kawahara and Barber  
179 (18)). In our data set, we find preliminary evidence of independent origins of sonar  
180 jamming in at least six moth subfamilies (Sphinginae, Macroglossinae, Aganainae,  
181 Arctiinae, Calpinae, Lymantriinae) based on this threshold. A seventh subfamily  
182 (Smerinthinae) also independently developed duty cycles capable of jamming, yet they  
183 are not capable of this behavior as this group lacks ears and thus cannot respond in  
184 advance to attacking bats. Animals that use complex tymbals with multiple  
185 microstriations (aganines, arctiines, and lymantrids) and stridulatory mechanisms  
186 (calpines and sphingids) are also likely capable of jamming. Thus, although moth  
187 morphology is not strictly deterministic of sound production function, some  
188 morphologies (wing beating mechanisms and tymbals with few microstriations; (44))



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189 cannot support the high duty cycle (and likely high intensity) sounds necessary for  
190 jamming (18, 22).

191         Sonar jamming appears to be a derived strategy that has arisen repeatedly and  
192 recently in multiple lineages. Our preliminary investigations indicate that this strategy is  
193 not uniformly related to a loss (or lack of gain) of unpalatability to bats. We find that  
194 some genera capable of jamming bat sonar are palatable (Dataset S2; see Methods for  
195 palatability experimental details) and other genera are not, sometimes within the same  
196 subfamily (Arctiinae and Lymantriinae), thus the hypothesis that the origin of duty cycles  
197 capable of jamming frees lineages from the costs of sequestering chemicals for protection  
198 against bats (45) seems unlikely to be commonly supported. One possibility is that  
199 hostplant specialization canalizes sequestration strategies. Advertising difficulty of  
200 capture (evasive aposematism) is another conceivable function of conspicuous high duty  
201 cycle sounds (46) that may operate alongside sonar jamming, however, this hypothesis  
202 remains untested.

203         It appears that most sound-producing moths are not capable of jamming bat sonar.  
204 The majority of sound producers are therefore likely communicating with their bat  
205 predators, rather than disrupting echolocation. We found that moth genera that produced  
206 anti-bat sounds were commonly split between those that were palatable to bats and those  
207 that were not. Geometrid moths indeed seemed to be noxious, but not as repellent as  
208 lymantrids or arctiines (Dataset S2). Multiple subfamilies (Calpinae, Erebinae,  
209 Noctuidae, Nystaleinae, Macroglossinae, Smerinthinae, and Sphinginae) were considered  
210 quite palatable by the bats we pit these moths against (see supplement). These results

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211 likely indicate that these animals are exploiting the education imparted to their predators  
212 by unpalatable models (i.e., they are Batesian mimics).

213         To test the possible organization of anti-bat sounds into acoustic mimicry rings,  
214 we intensively studied a community of moths in Sumaco, Ecuador. We captured moths  
215 with UV lights and queried this megadiverse community for anti-bat acoustic response  
216 over 14 continuous nights. To analyze the resulting acoustic data, we used a  
217 dimensionality reduction algorithm (UMAP: Uniform Manifold Approximation and  
218 Projection; (47)) to find groups of moths with similar acoustic features (clusters). This  
219 unsupervised machine-learning algorithm estimates the topology of high dimensional  
220 data and uses this information to build a low dimensional representation that preserves  
221 relationships present in the data. We used 10 acoustic features (see Methods) and 33  
222 species as input to UMAP to project the data from a 10-dimensional space into a 2D  
223 space where we found five well-separated clusters (Fig. 3; interactive 3D visualization at:  
224 [http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/p](http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json)  
225 [oli/main/ec6.json](http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json)).

226         While we caution that this analysis offers only a cursory temporal and spatial  
227 snapshot of the hyper-diverse mimetic associations that are likely present, we find some  
228 remarkable patterns. Each cluster of moth anti-bat sounds includes at least one species  
229 that we have found to be unpalatable to bats and most clusters also contain animals that  
230 bats readily consume. For example, one acoustic cluster contains one unpalatable dalcerid  
231 (Dalceridae), five palatable calpines (Erebidae: Calpinae), and two palatable sphingids  
232 (Sphingidae: Macroglossinae). Another cluster consists of six geometrid species  
233 (Ennominae) and one tiger moth (Erebidae: Arctiinae) all of which are likely honestly

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234 advertising noxious taste - perhaps a Müllerian ensemble. Interestingly, one cluster of  
235 Arctiini tiger moths (Erebidae: Arctiinae) uniformly contains extremely high duty cycle  
236 species capable of jamming bat sonar, including two genera that appear to be unpalatable  
237 to bats, supporting the prediction that jamming and aposematism are not mutually  
238 exclusive (27). Our preliminary data portends substantial community-level structuring of  
239 ultrasonic warning signals driven by the psychologies of syntopic bat predators (48). We  
240 are at the frontier of understanding a hidden dimension of biodiversity – the ultrasonic  
241 information transfer between bats and their insect prey.

242       Importantly, many species of moths also use ultrasonic sounds to transmit  
243 information to conspecifics – with males from at least six families (Crambidae, Erebidae,  
244 Geometridae, Noctuidae, Pyralidae, and Sphingidae) likely using this strategy to attract  
245 mates (49, 50). Some male moths use intense ultrasonic signals to communicate with  
246 females, as in tiger moths (Erebidae: Arctiinae) (50). Other families of moths produce  
247 quiet mating calls (Noctuidae, Arctiidae, Geometridae and Crambidae), apparently  
248 intended for nearby females (50). These “whispering” moths likely employ soft signals to  
249 avoid detection by eavesdropping bats and other predators (51–53). It is unclear if the use  
250 of ultrasound by moths evolved first in a mating context, or if it was secondarily co-opted  
251 from an anti-bat origin. Some moths are able to discriminate mates from bats, such as  
252 *Achroia grisella* (Pyralidae) females that exhibit differing behaviors, positive phonotaxis  
253 or freezing, when stimulated by different pulse rates (higher pulse rate indicating a  
254 conspecific calling male and lower pulse rate indicating an approaching bat, respectively  
255 (54). Alternatively, female *Spodoptera litura* (Noctuidae) are unable to distinguish  
256 attacking bats from ultrasound-producing males, suggesting a sensory exploitation origin

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257 of sound production in moths – that is, male moths exploit female freezing behavior to  
258 secure matings (55). We do not yet know whether moths that acoustically respond to  
259 echolocating bats are more likely to use ultrasound for mating, as many moths have not  
260 yet been tested for these behaviors (56), but this notion seems likely.

261        Ultrasonically-signaling moths appear to be connected by some of the most  
262 widespread and biodiverse mimicry complexes known to date (57, 58). The dynamics of  
263 these associations stand as a great unknown in natural history, and a laboratory for  
264 understanding mimicry dynamics and convergent evolution (59). The intense pressure to  
265 thwart the attacks of echolocating bats seems to have also driven ultrasound production in  
266 other insects. Tiger beetles (Cicindelidae) produce ultrasonic warning signals in response  
267 to sonar playback (60) and fireflies (Lampyridae: Lampyrinae), known to be noxious to  
268 bats (61), constantly produce ultrasonic clicks in flight, which may serve as a component  
269 of a multi-modal aposematic signal to bats (62). We predict that a complete  
270 understanding of ultrasonic mimicry rings will involve a thorough analysis of all major  
271 nocturnal, aerial insect groups including moths (Lepidoptera), beetles (Coleoptera), true  
272 bugs (Hemiptera), flies (Diptera), lacewings and antlions (Neuroptera) and more.  
273 Understanding how bat receivers generalize the massive numbers of insect warning  
274 sounds into categories is an important frontier in understanding this powerful selective  
275 force. Bats have shaped the nocturnal soundscape in profound ways – driving a chorus of  
276 nightly cries, across the globe, as moths and perhaps other insects jam sonar, warn of  
277 noxious chemicals, and mimic the sounds of unpalatable models. Comprehending this  
278 symphony is central to understanding insect biodiversity.

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### 299 **Author Contributions**

300 JRB and AYK designed and supervised the research and led all fieldwork. All authors  
301 collected data. DP led the phylogenetic analysis with input from AYK, JRB. MN led the  
302 machine learning analyses with input from JRB. JJR and KAM led moth sound analysis

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303 with input from JRB and assistance from BQ-R. DP and NTH led moth specimen  
304 identification. JRB and JJR wrote the first draft of the manuscript. All authors contributed  
305 to writing.

### 306 **Data and Materials Availability**

307 The newly sequenced DNA barcodes used in this study have been deposited in the  
308 National Center for Biotechnology Information's GenBank sequence database (all  
309 accession nos. provided in Dataset S4). All other data are available in the main text, the  
310 Supplementary Information, or at the Dryad Digital Repository (link to come when  
311 published).

### 312 **Figure Legends**

313 **Figure 1.** A molecular phylogeny of Lepidoptera indicating anti-predator ultrasound  
314 production across the order. Bars and nodes with magenta outlines represent taxa  
315 associated with sufficiently large duty cycle values (>18%) for sonar jamming. Asterisks  
316 indicate taxa known to produce ultrasound, but not in response to either tactile stimuli nor  
317 bat ultrasound. Grayscale images indicate taxa that do not produce ultrasound. This  
318 phylogeny is meant to illustrate the diversity of ultrasound production and offer broad  
319 strokes on the origins of anti-predator sounds at the family and subfamily level, not as a  
320 test of evolutionary relationships. Photographs are distributed under Creative Commons  
321 Attribution NonCommercial Licenses (see Fig. S2, Dataset S3 for full accreditations).

322 **Figure 2.** Anti-bat ultrasound-producing structures. A-D. *Mittonia hampsoni* (Pyralidae:  
323 Pyralinae) produces ultrasonic clicks in flight via modified scales on the tegula; A. Scale  
324 bar = 1.0 cm; B. Tegula, 0.2 mm; C. Tegular scales, 50  $\mu$ m; D. Response to bat sonar  
325 playback (*Mittonia hampsoni*), 100 ms). E-H. *Lymantria sp.* (Erebidae: Lymantriinae)

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326 generates ultrasound with paired tymbals recessed in abdominal pockets; E. Scale bar =  
327 1.0 cm; F. Arrow indicates one of the tymbal pair, 1.0 mm; G. Close up of one tymbal,  
328 0.5 mm; H. Response to bat sonar playback (*Lymantria sp.*), 100 ms. I-L. *Melese sp.*  
329 (Erebidae: Arctiinae) emits ultrasound with paired thoracic tymbals; I. Scale bars = 1.0  
330 cm; J. Tymbal 0.5 mm; K. Close-up of microstriations on tymbal surface, 0.1 mm; L.  
331 Response to bat sonar playback (*Melese peruviana*), 100 ms. M-P. *Gonodonta sicheas*  
332 (Erebidae: Calpinae) produces ultrasound by stridulating modified abdominal scales; M.  
333 Scale bar = 1.0 cm; N. Patch of stridulatory scales, 0.5 mm; O. Stridulatory scale, Scale  
334 bar = 50  $\mu$ m; P. Response to bat sonar playback (*Gonodonta bidens*), 100 ms. Q-T.  
335 *Xylophanes falco* (Sphingidae: Macroglossinae) produces ultrasound by stridulating  
336 modified genital valves; Q. Scale bar = 1 cm; R. Patch of stridulatory scales on genital  
337 valve, 0.5 mm; S. Stridulatory scales, 0.2 mm; T. Response to bat sonar playback  
338 (*Xylophanes amadis*), 100 ms.

339 **Figure 3.** Purported acoustic mimicry rings of a community of moths in Sumaco,  
340 Ecuador (33 species). A UMAP (Uniform Manifold Approximation and Projection)  
341 projection shows clusters of moth anti-bat sounds with similar acoustic features. The  
342 relative distance between the clusters is meaningful in the sense that clusters that are  
343 close in the 2D map, are more similar than clusters that are further away. Photos of moths  
344 are congeners at the genus level. All photos taken by the authors. *Xylophanes titana*,  
345 purple diamond, solid circle; *Gonodonta syrna*, grey diamond, open circle; *Scaptius*  
346 *ditissima*, green sun, solid circle; *Melese sordida*, green sun, open circle; *Agylla sp.*,  
347 green triangle, solid circle; *Acraga moorei*, dark-blue triangle, open circle; *Bertholdia*  
348 *bilineola*, green square, solid circle; *Melese chozeba*, green square, open circle; *Eucereon*

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349 *formosum dognini*, green star, solid circle; *Nephodia* sp., blue star, open circle. See

350 Supplement Archive 11 for palatability data at the genus level.

### 351 **Methods**

#### 352 **Statement on Fieldwork Ethics**

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

#### 360 **Echolocation playback, tactile stimulation, and acoustic recording**

361 We assayed moths in three of the world's tropics: South America (Ecuador, French  
362 Guiana), Africa (Mozambique), and Asia (Malaysian Borneo) for ultrasonic reply to  
363 handling and bat attack. To simulate handling by a predator, we lightly compressed the  
364 moth's head, abdomen, or thorax. We simulated bat attack using six recorded bat  
365 echolocation attack sequences (see supplement). Bat assemblages and echolocation  
366 strategies vary across the world. To capture some of the diversity of echolocation calls  
367 that moths might experience in different tropical regions, we presented moths with three  
368 different frequency modulated (FM) echolocation attacks and two constant frequency  
369 (CF) attacks. Two of the FM sequences were recorded from trained bats attacking a moth  
370 tethered 10 cm from a microphone (FM1: *Lasiurus borealis*, FM2: *Eptesicus fuscus*) (19).  
371 We also generated a synthetic bat attack based on the short-duration, broadband



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372 echolocation cries of some bats (63) (synthetic). To represent CF bat calls, we used on-  
373 board telemike recordings of bats (*Rhinolophus ferrumequinum nippon*) attacking prey  
374 provided to us by Yuki Kinoshita and Shizuko Hiryu (64) (CF1, CF2). All bat calls were  
375 played through an Avisoft UltraSoundGate Player BL Pro Speaker/ Amplifier ( $\pm 6$  dB,  
376 20-110 kHz, playback sampling rate 250 kHz) placed 10 cm behind the moth's abdomen,  
377 except in the cases of sphingid moths, where the speaker was positioned on-axis 10 cm  
378 from the moth's face, as their hearing organs are comprised of their mouthparts (65).  
379 Similarly, we recorded moth sounds using an Avisoft CM16 condenser microphone ( $\pm 3$   
380 dB, 20-140 kHz) attached to an UltraSoundGate 116Hme DAQ sampling at 375 kHz via  
381 a laptop computer running Avisoft Recorder software, placed at a 90° angle 10cm from  
382 the moth's thorax, except in the cases of sphingid moths, where the microphone was  
383 placed 10 cm directly behind the moth (as the genitals were previously known as the  
384 sound-producing organs in this group (19)).

385       Regardless of mechanism of ultrasound production, we focused our analyses on  
386 one complete modulation cycle of sound, which we defined as the two-component  
387 structure of the sound emissions. This paired structure results from: 1) the up-down wing  
388 stroke, 2) the buckling-unbuckling of tymbals, 3) the in-out or side-side stridulating of  
389 valves. We used Avisoft SASLab Pro software to measure three modulation cycles from  
390 each individual in our data set, except in cases where only two could be measured. We  
391 extracted the same parameters as those described in Barber & Conner (27) for  
392 comparability to other studies. To measure the temporal characteristics – duty cycle  
393 (proportion of 100ms window with moth sound present), duration of modulation cycle,  
394 and duration of modulation cycle components – we used the pulse train analysis tool with

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395 the following settings (Time constant=0.025ms, Threshold=0.15V, Hysteresis=15dB,  
396 Start/end threshold=-15dB, Envelope=Rectification + exponential decay, Pulse  
397 detection=Peak search with Hysteresis). We measured spectral characteristics – dominant  
398 frequency, frequency 15 dB above and below dominant frequency – from the Power  
399 Spectrum (averaged) tool with a Hann evaluation window and FFT=1024.

400 We attempted to record as many specimens as possible of each moth species,  
401 though this was usually limited by the number of healthy specimens we encountered in  
402 the field. For downstream analyses, we only considered a species to be responsive (i.e.,  
403 producing ultrasound in response to bat ultrasound and/or tactile stimuli) if we recorded  
404 responsive ultrasound production in at least two specimens. Otherwise, the recorded  
405 species were assumed to be non-responsive. This is not the preferred method for  
406 obtaining negative data, since it is plausible that a moth could be capable of responding to  
407 stimuli, yet did not do so in our setting. However, we believed it was necessary to  
408 delineate between moths actually observed in the field, and moths that we were unable to  
409 test at all, but that were incorporated into our phylogeny. Thus, the non-responsive moths  
410 in the field were treated as having negative data, whereas the untested moths were treated  
411 as having missing data (see Phylogenetic Methods).

### 412 **Palatability**

413 Palatability experiments were conducted on 93 moths from 26 species (see supplement)  
414 in the field. We ablated sound-producing structures (if present), before offering a hand-  
415 held captive bat (see supplement for species and locations) a moth via forceps. In an  
416 attempt to control for the foraging motivation of each bat, we only scored interactions  
417 where the bat was willing to eat a control moth (a species we knew to be palatable) both

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418 before and after we offered an experimental moth. We scored partial palatability by  
419 dividing the length of the moth body into six parts and assigning one point to the head,  
420 two points to the thorax, and three points to the abdomen, following the methods of  
421 Hristov and Conner (42). A palatability score of 0 indicates the moths was entirely  
422 rejected and a score of 6 indicates the moth was 100% consumed.

### 423 **Unsupervised machine learning cluster analysis of moth sounds**

424 The dimensionality reduction algorithm Uniform Manifold Approximation and Projection  
425 (UMAP) (47) was used for finding groups of moth sounds with similar features (clusters).  
426 Dimensionality reduction algorithms capture variability in a limited number of random  
427 variables to allow two or three-dimensional visualization of data that resides in a  
428 multidimensional space. The most common approach is the method of principal  
429 component analysis (PCA) (66), which uses linear combinations of variables to generate  
430 orthogonal axes that capture the variation present in the data with fewer variables.  
431 Another approach, developed a century after PCA, t-Distributed Stochastic Neighbor  
432 Embedding (t-SNE) (67), carries out dimensionality reduction by analyzing similarity of  
433 points using a Gaussian distance in high dimensional space and mapping these data into a  
434 low dimensional space. t-SNE is able to capture local non-linear relationships in the data,  
435 which PCA by its linear design is not able to, but does not capture the global structure. A  
436 more recent method, UMAP, is an unsupervised machine-learning algorithm for  
437 dimension reduction based on manifold learning techniques and ideas from topological  
438 data analysis. It works by estimating the topology of the high dimensional data and uses  
439 this information to build a low dimensional representation that preserves relationships

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440 present in the data. It is better at mapping the global structure of the data from the high  
441 dimensional space than t-SNE, and is able to capture local relationships as well.

442 We used the moth acoustic features to define a multidimensional space where  
443 each moth is represented by a vector (or point) in that space. The data set consisted of 33  
444 entries with 10 features each which translates to 33 points (vectors) in a 10-dimensional  
445 space. We input their coordinates into a PCA as a pre-processing step. The resultant  
446 principal components were then used as input to UMAP to project the data from the 10-  
447 dimensional space into a 2D space. Each cluster shares similar features. The relative  
448 distance between the clusters is meaningful in the sense that clusters that are close in the  
449 2d map, are more “similar” than cluster that are farther away. The features variables used,  
450 extracted from audio files, were "MC DC mean", "d MC mean", "D 1/2 mean", "D silent  
451 mean", "D 2h mean", "DF mean", "D dB mean", "+ 15 dB mean", "- 15 dB mean", "100 ms  
452 DC mean" (see supplement for definitions). We used the software tools Scikit-learn (68)  
453 and pandas (69). The steps of dimensional reduction using the different methods we have  
454 discussed above can be seen in the interactive online version of the embedding  
455 ([http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/p](http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json)  
456 [oli/main/ec6.json](http://projector.tensorflow.org/?config=https://raw.githubusercontent.com/nunezmatias/poli/main/ec6.json)) by clicking on the different bookmarks on the right (created via (70)).

### 457 **Phylogenetic methods**

458 In order to determine the timing of evolution of anti-bat sound production in Lepidoptera,  
459 we created a dated molecular phylogeny, using the ages estimated in the Lepidoptera  
460 phylogeny of Kawahara et al. (12), that incorporates the moth taxa we tested for anti-bat  
461 ultrasound production. We attempted to find previously published COI barcodes and five  
462 commonly sequenced nuclear genes (CAD, DDC, EF1-A, period, wingless) for one

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463 species of every genus that was tested for anti-bat sound production (as well as the  
464 sound-producing genus tested in Corcoran and Hristov (20), and also used published data  
465 from as many species as possible that were included in the Kawahara et al. (12) dataset  
466 (this transcriptomic dataset lacked data for these six genes and thus could not directly be  
467 used). Whenever possible, molecular data for a genus was represented by a tested  
468 species; when such data were not available (after searching both NCBI and Bold  
469 Taxonomy Browser), a congener was used instead.

470         There were 11 genera from our sound production dataset that had no available  
471 sequence data; in order to represent these taxa in our analysis, we obtained new COI  
472 barcodes from DNA extracted from the legs of the ensouffled specimens. DNA was  
473 extracted using an OmniPrep Genomic DNA Extraction Kit (G-Biosciences, St. Louis,  
474 MO), following the protocol of Espeland et al. (71) and PCR was performed following  
475 the protocol of Hebert et al. (72) using Lep1 reverse primers. Sanger sequencing was  
476 performed by Genewiz (South Plainfield, NJ). COI sequencing was unsuccessful for two  
477 non-sound-producing genera (*Grammodora*, *Trotonotus*), which were consequently  
478 excluded from the analysis. The nine newly sequenced barcodes used in this analysis  
479 were uploaded to NCBI ([GenBank IDs to be added after acceptance]), and specimen  
480 vouchers were deposited at the McGuire Center for Lepidoptera and Biodiversity  
481 (MGCL; Dataset S4). In total, our molecular dataset contained at least one gene for 432  
482 Lepidoptera species.

483         Sequences for the six genes were aligned in MAFFT (73), then manually trimmed  
484 and concatenated in GENEIOUS v.11.1.5. The dataset was partitioned by codon position,  
485 constrained using the topology in figure 1 of Kawahara et al. (12), and a maximum

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486 likelihood analysis was performed in IQ-TREE v.1.6.2 (74), using ModelFinder to  
487 determine the best-fit substitution models for each partition (75). The resulting maximum  
488 likelihood tree was dated in TreePL (76), using the age estimates from Kawahara et al.  
489 (12) as secondary calibrations. The molecular dataset and other files associated with these  
490 analyses are included in Supplementary Archives 1–9.

491 Two ancestral state reconstructions (ASRs) of anti-bat sound production were  
492 performed using stochastic character mapping with the ‘make.simmap’ in the R package  
493 Phytools v07-70 (77). Symmetrical transition rate models were used in both ASRs, and  
494 1000 simulations were performed. In order to reduce the amount of computational  
495 resources required, these ASRs were performed only on the Ditrysia clade of the dated  
496 tree, which comprise 93% of all taxa in the analysis (400/432). Only one non-Ditrysiian  
497 genus had been tested for ultrasound production (Hepialidae: *Dalaca*, which did not  
498 produce ultrasound), so their absence did not significantly impact the ASR results since  
499 only 1/32 could have been confidently assigned a character state. In the first ASR, the  
500 evolution of anti-bat sound production was assessed by treating it as a ternary character,  
501 with taxa assigned to one of the following: 1. No sound production in response to a  
502 stimulus (this includes genera that constantly produce sound regardless of whether there  
503 is a stimulus, e.g. *Acraga*); 2. sound production in response to tactile stimuli; 3. sound  
504 production in response to both tactile stimuli and bat ultrasound (Dataset S5, Fig. S3). In  
505 instances where a species in the ensonified dataset was represented in the molecular  
506 dataset by a congener, we assumed that the congener had an identical character state. For  
507 taxa in the Kawahara et al. (12) dataset that were included in our ML analysis but not  
508 ensonified, an equal probability of 1/3 was assigned to each of the three states, if those

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509 taxa were known to have ears. For the untested taxa known to lack ears (12), we assumed  
510 they could not detect ultrasound and thus had no way to respond to bat calls, and we  
511 consequently assigned equal probabilities of 1/2 to the first two states, and 0 to the third  
512 state.

513 In the second ASR, the evolution of anti-bat sound production capable of  
514 jamming bat sonar (i.e., anti-bat ultrasound with a duty cycle value of at least 18%; (18)),  
515 was assessed by treating it as a binary character. Taxa were assigned to one of the  
516 following: 1. Duty cycle less than 18% (this includes genera that did not produce any  
517 ultrasound when tested); 2. Duty cycle of 18% or greater (Dataset S6, Fig. S4). As with  
518 the previous ASR, we assumed that congeners had identical character states. If duty cycle  
519 data were collected for multiple species in a genus, the value from the species with the  
520 largest mean duty cycle was used for that genus in the ASR (Supp. Archive 10). For  
521 untested taxa in the Kawahara et al. (12) dataset that were included in our ML analysis  
522 but not ensonified, an equal probability of 1/2 was assigned to each of the two states  
523 (regardless of whether they had ears). We also performed an ASR using maximum  
524 likelihood ('anc.ML' in Phytools v07-70 (77)), that modeled duty cycle as a continuous  
525 character (Dataset S7, Fig. S5). However, since this method cannot incorporate taxa with  
526 missing data, all non-ensonified taxa were assumed to have duty cycles of 0%.

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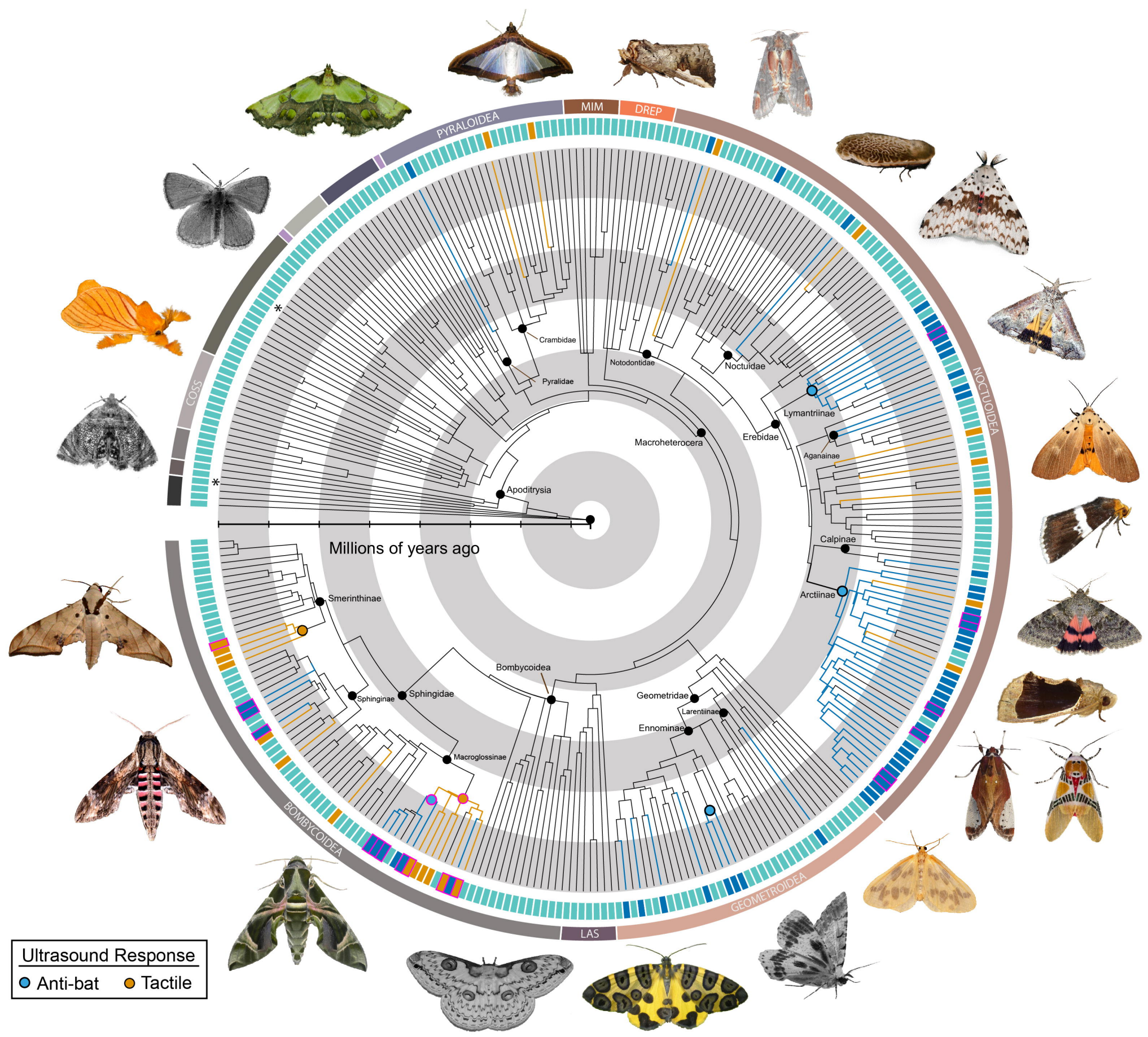
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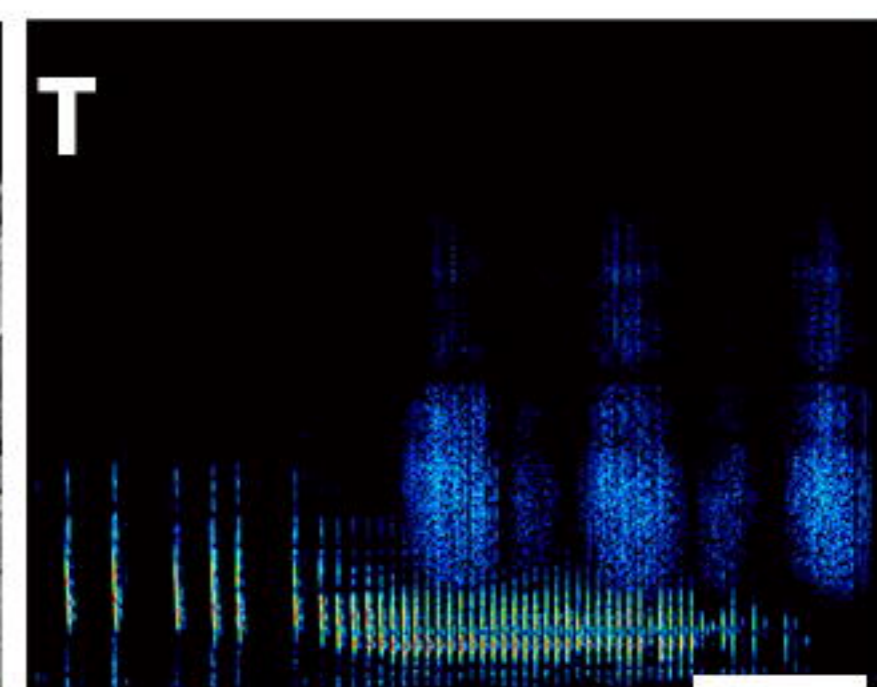
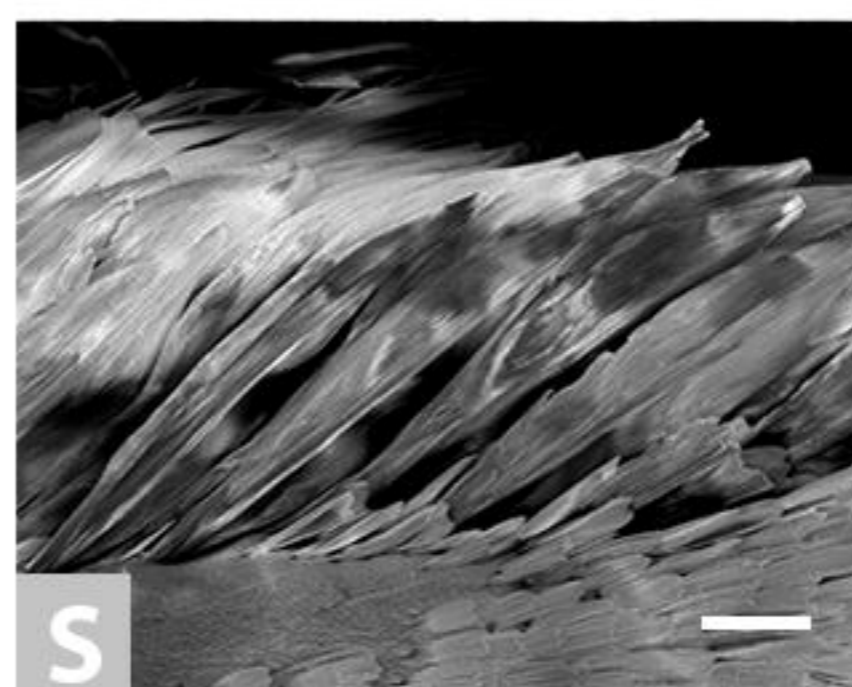
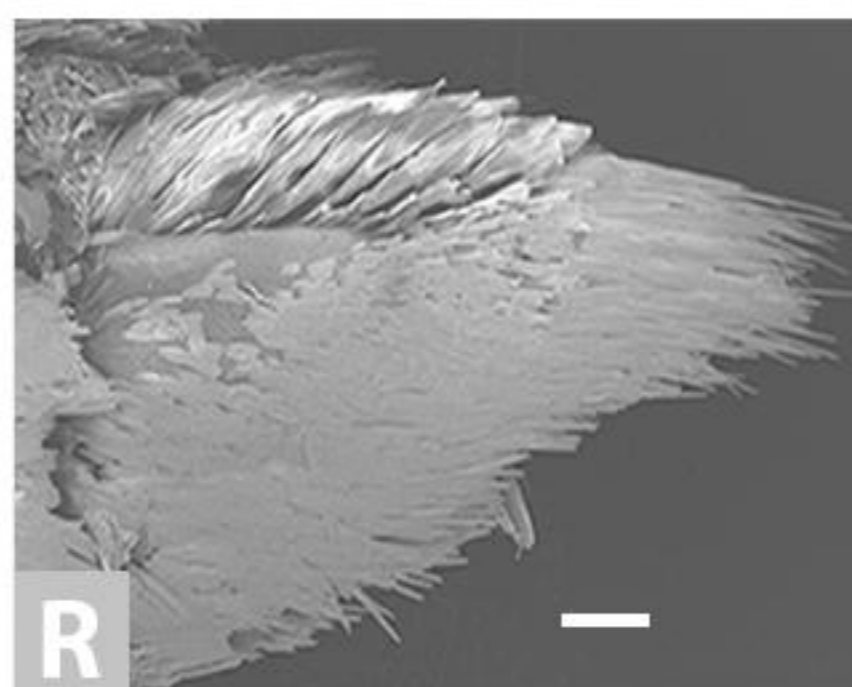
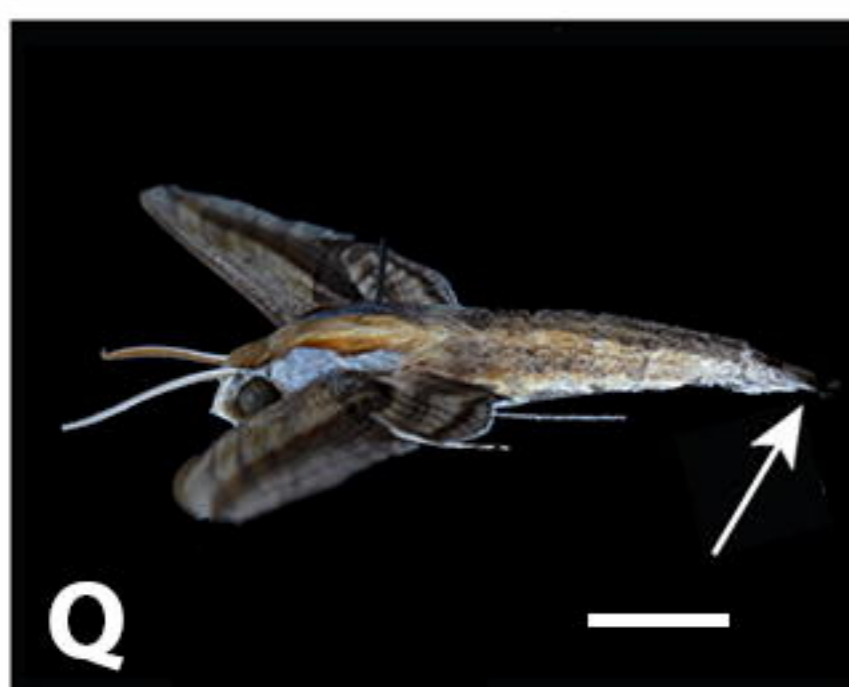
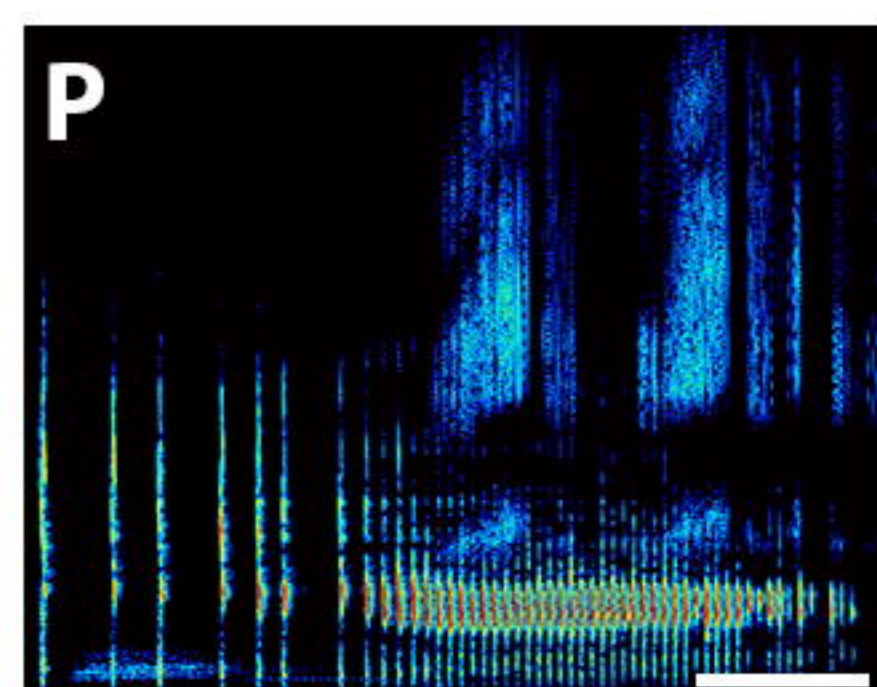
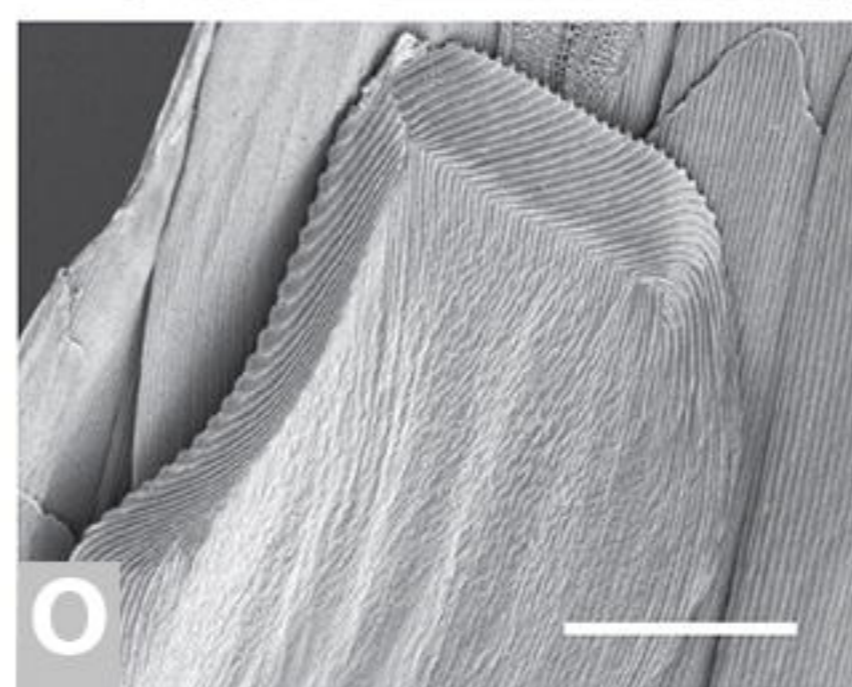
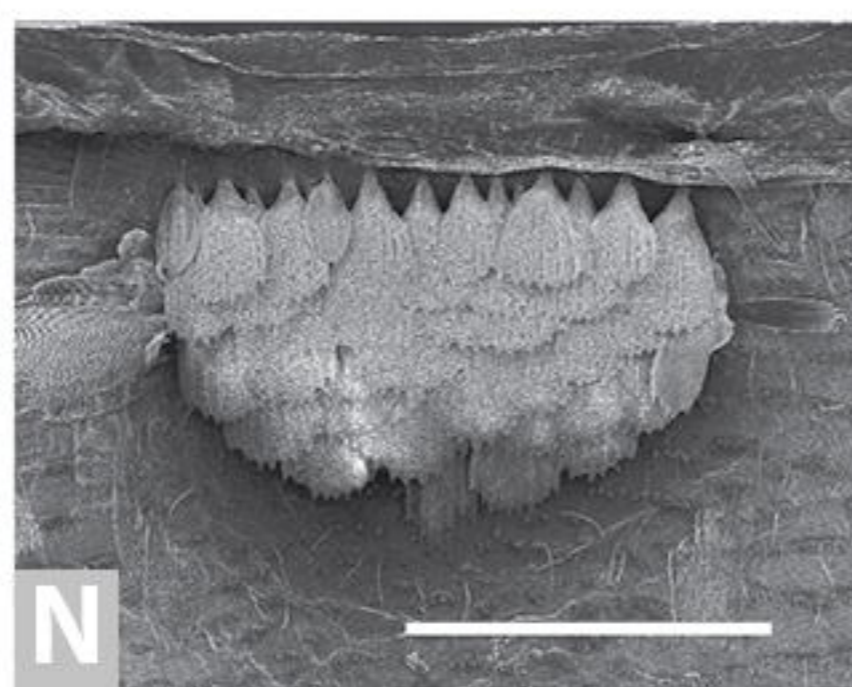
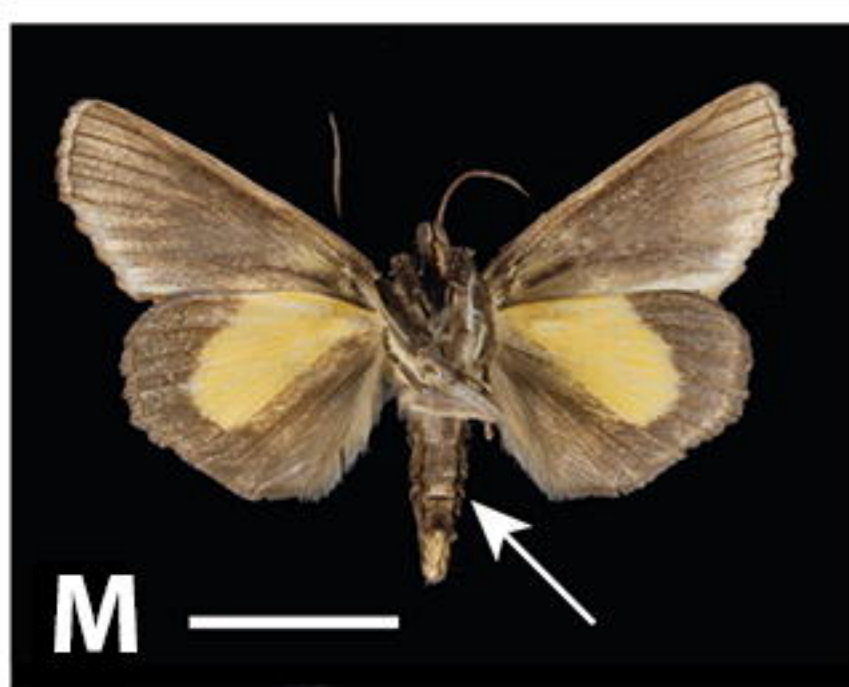
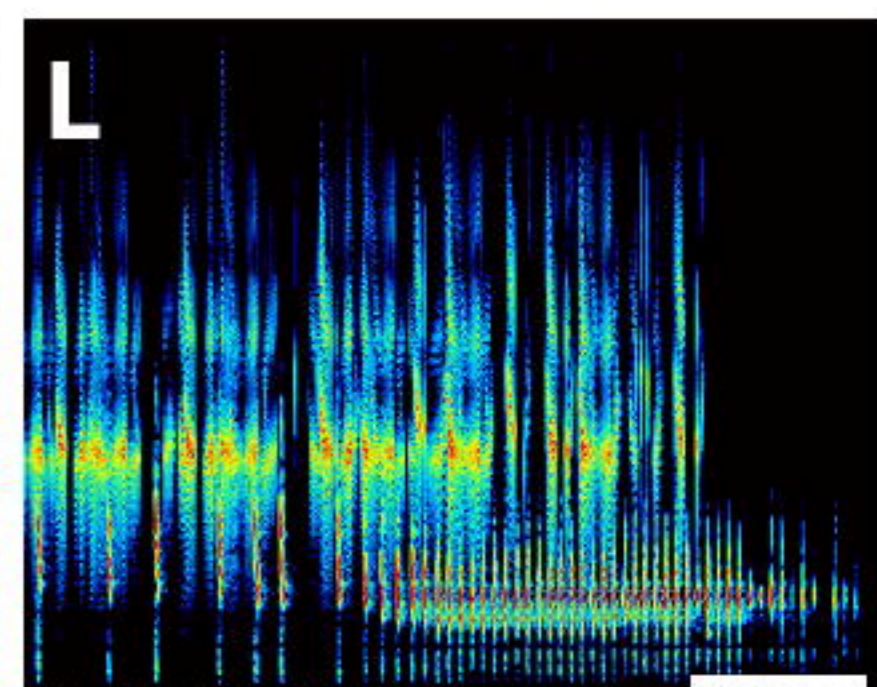
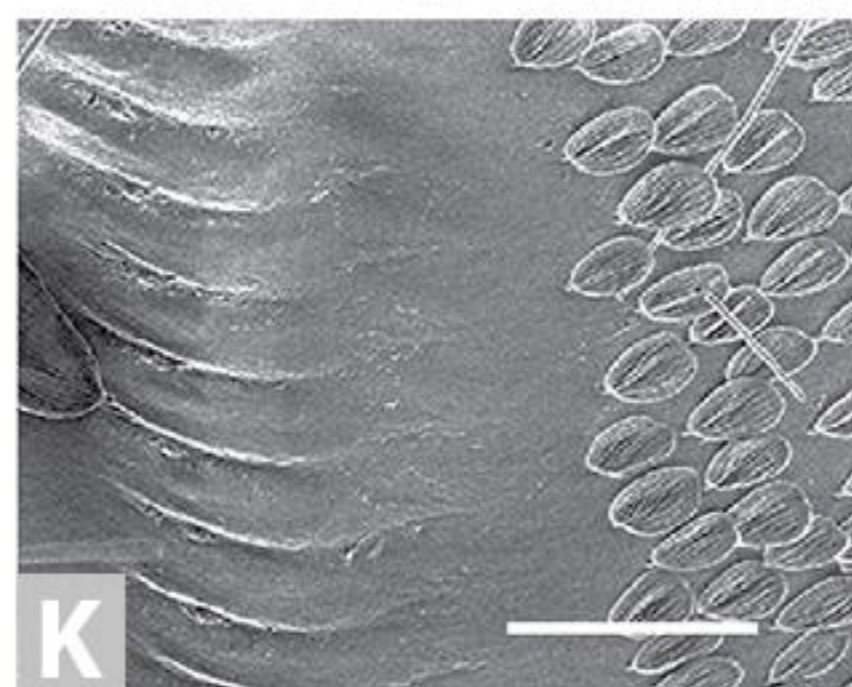
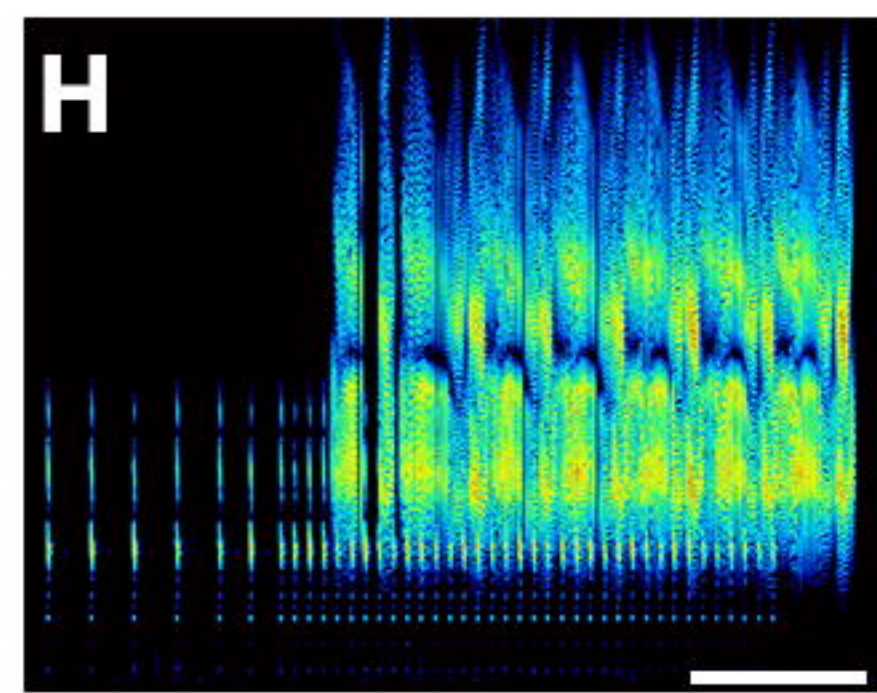
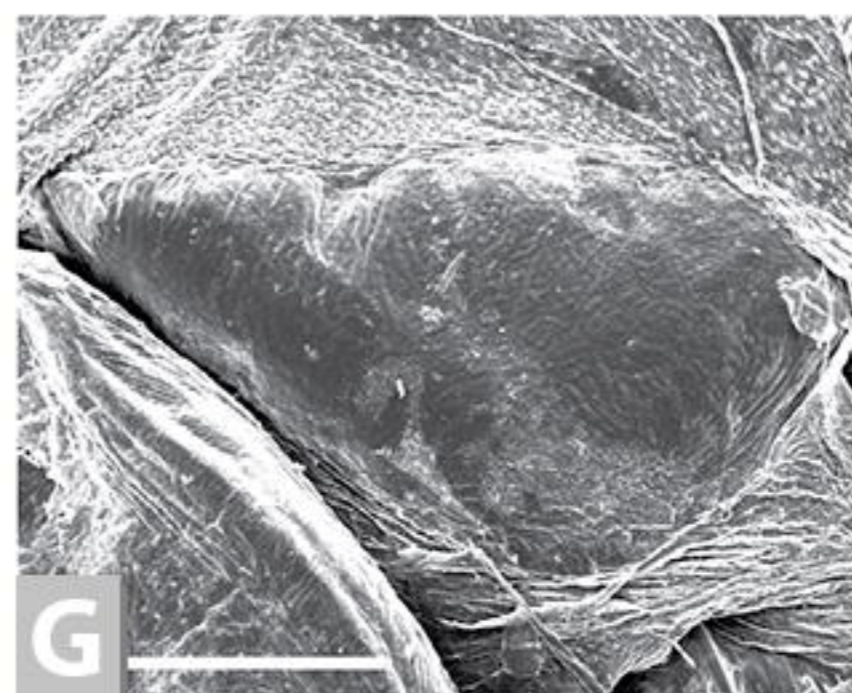
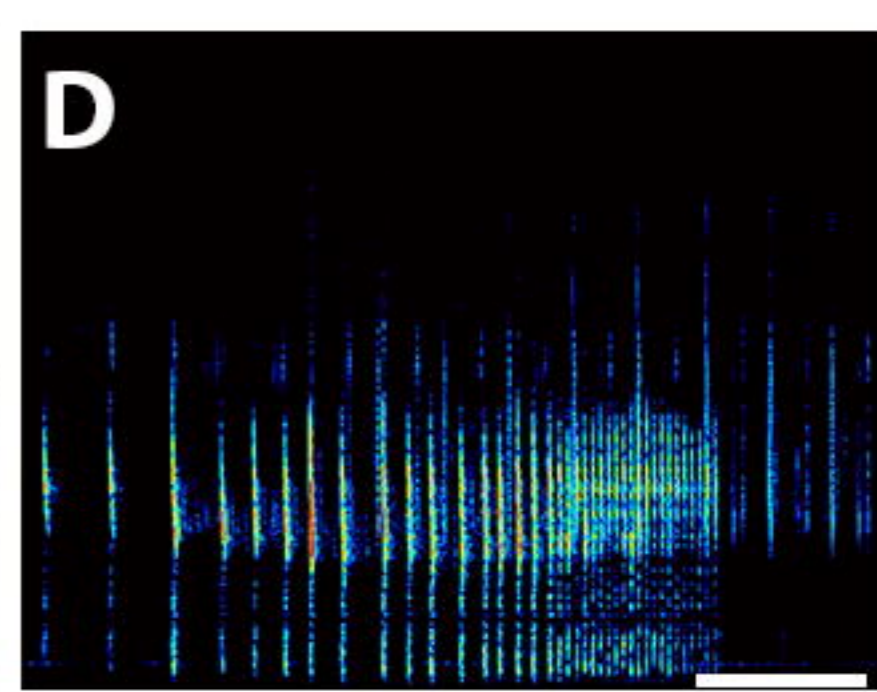
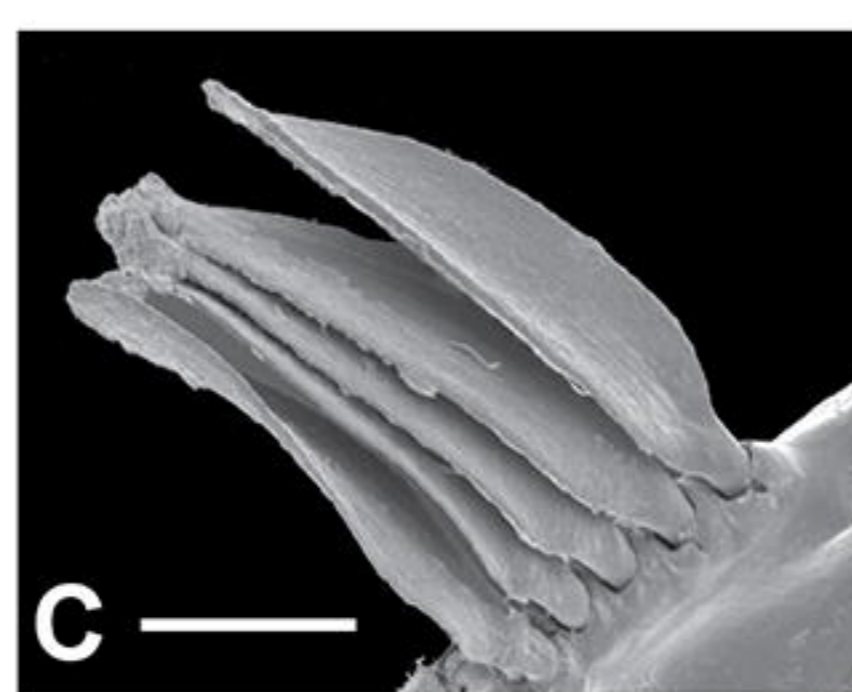
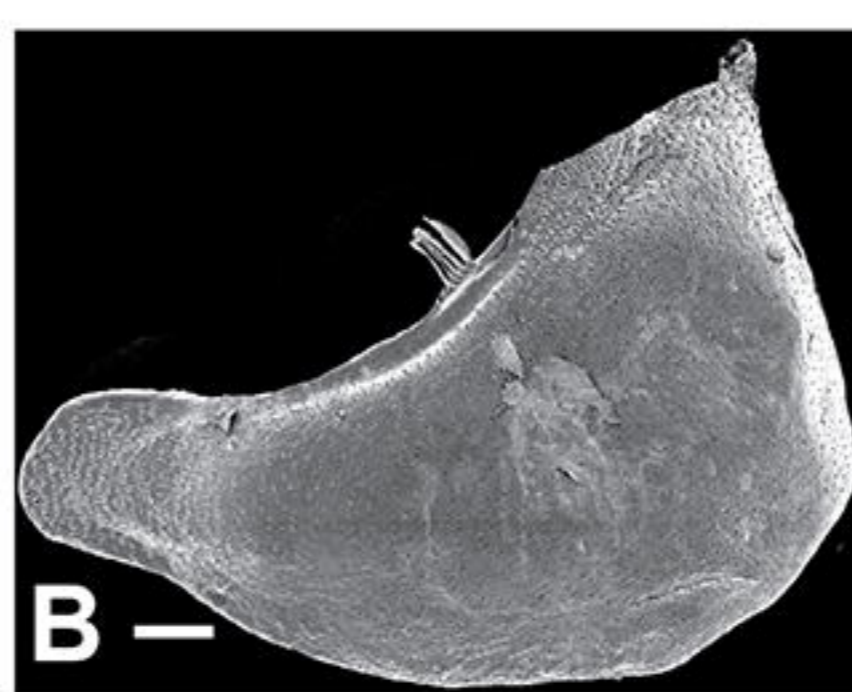
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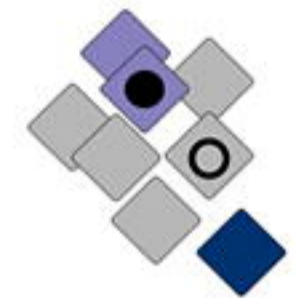
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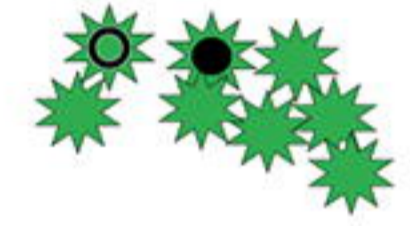




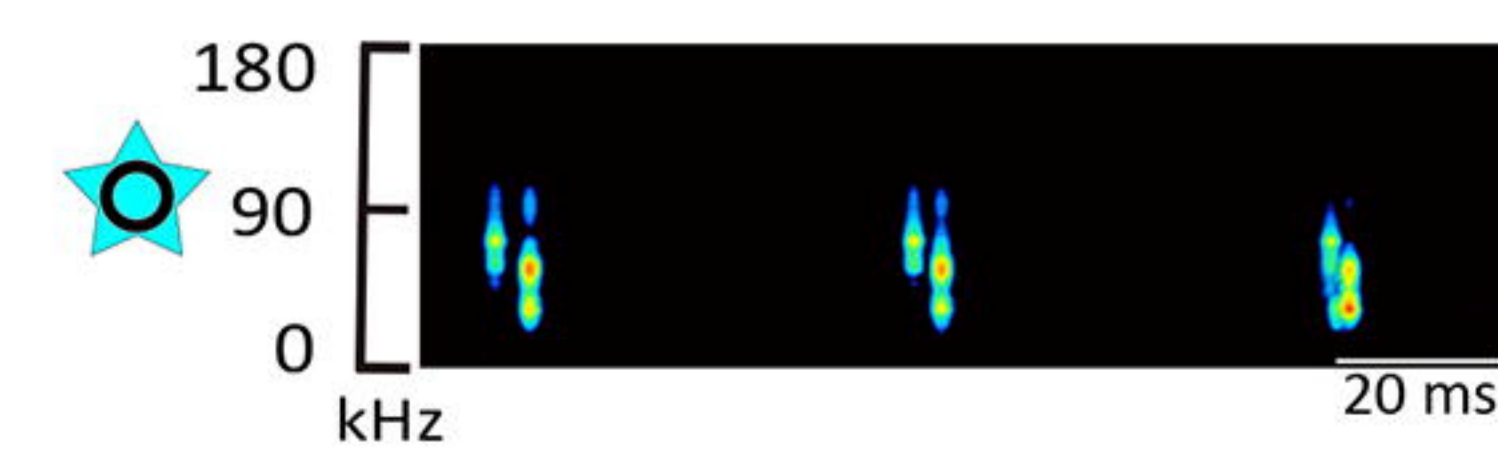
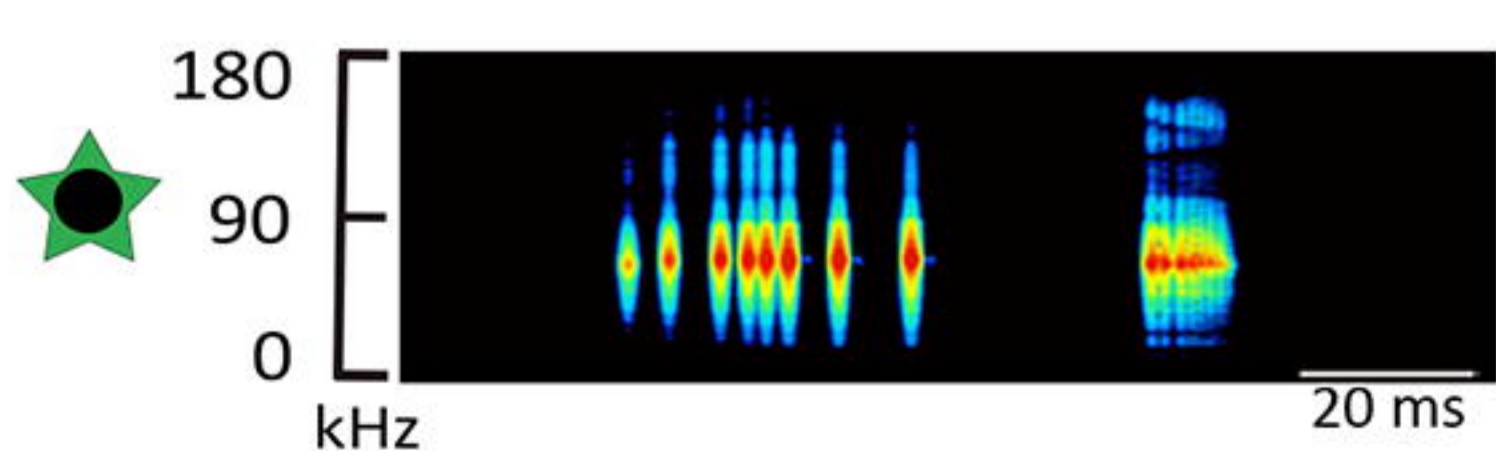
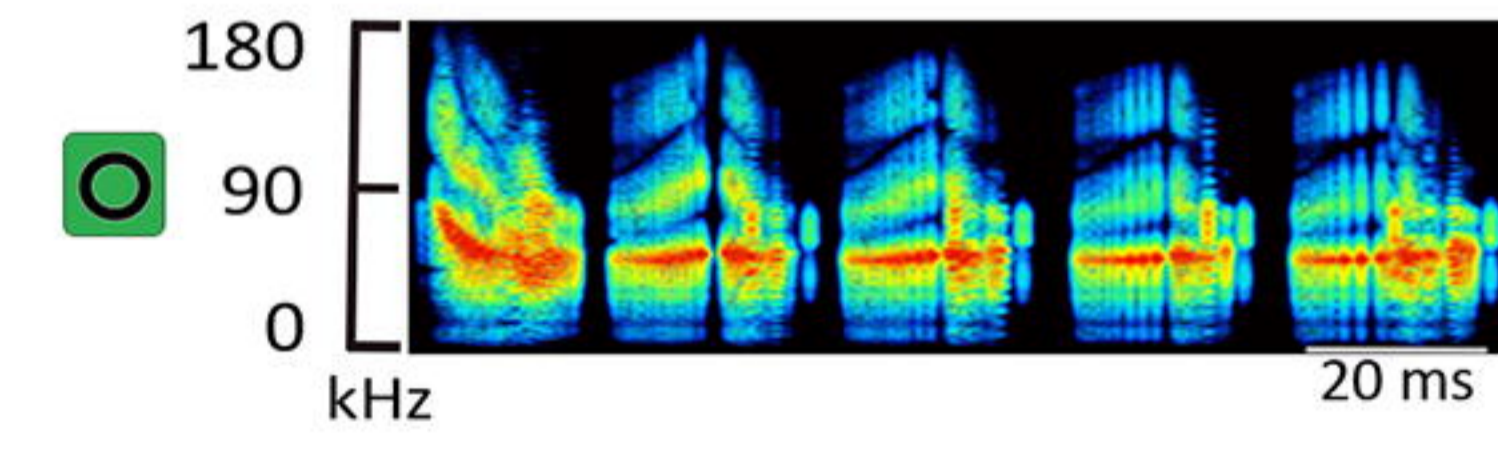
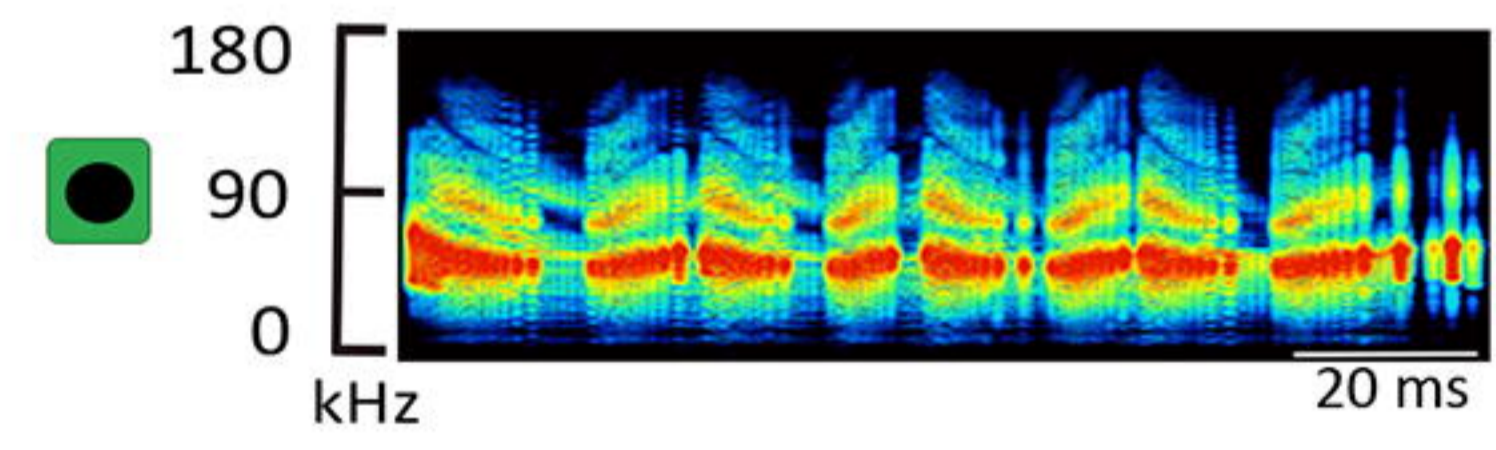
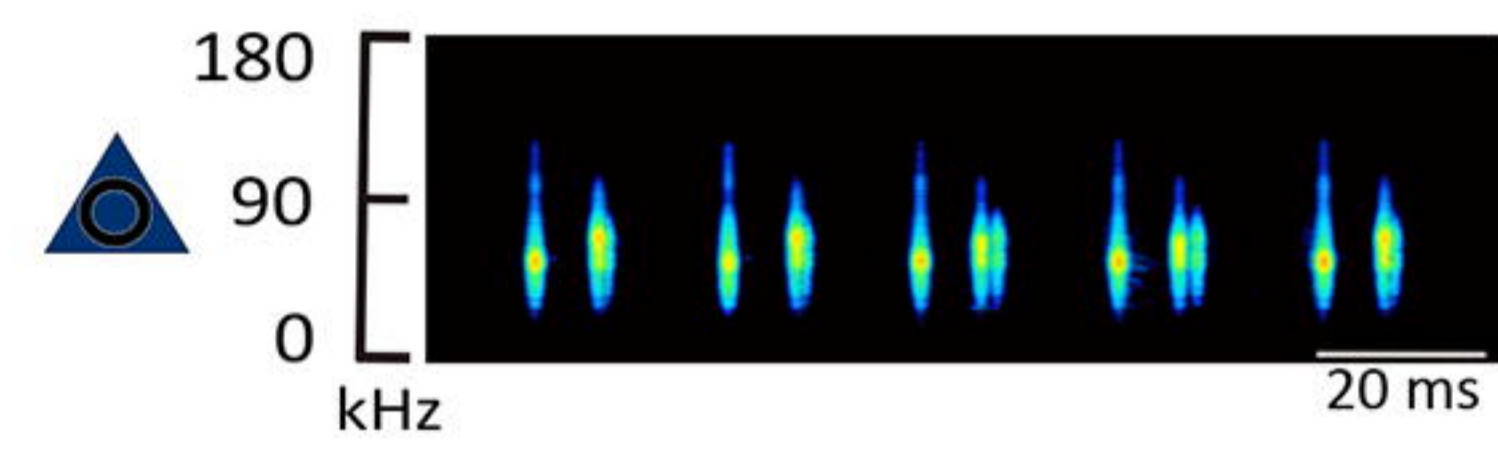
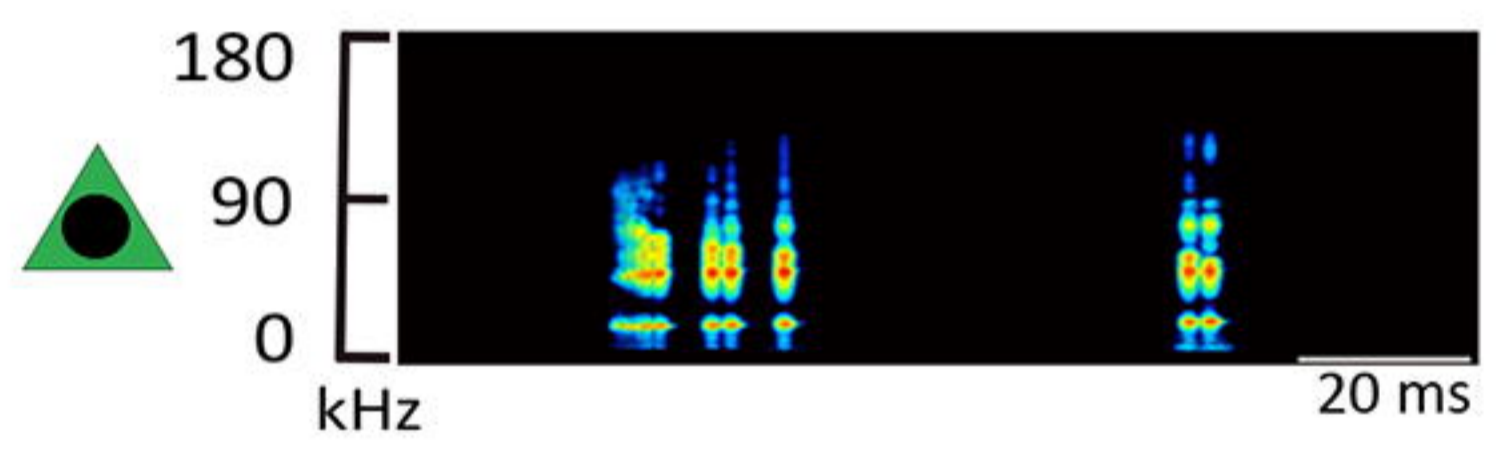
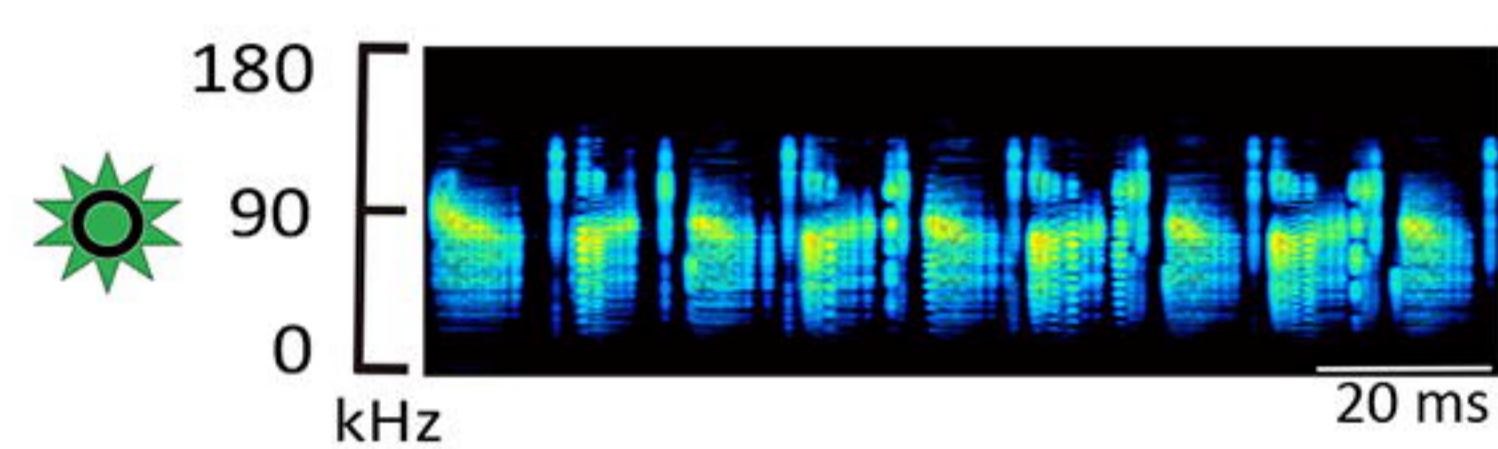
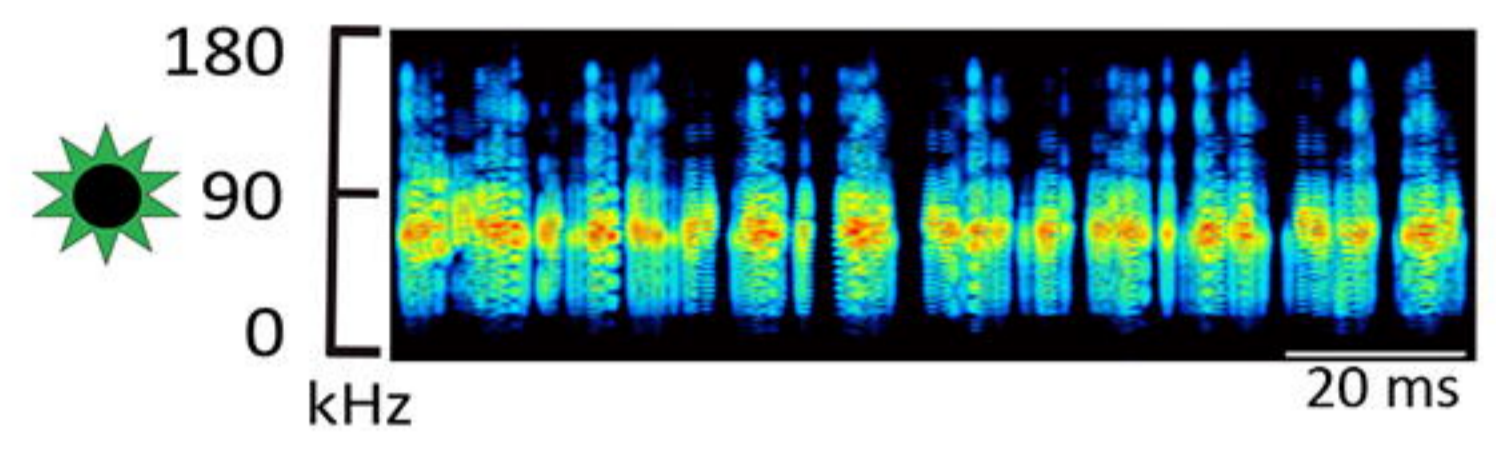
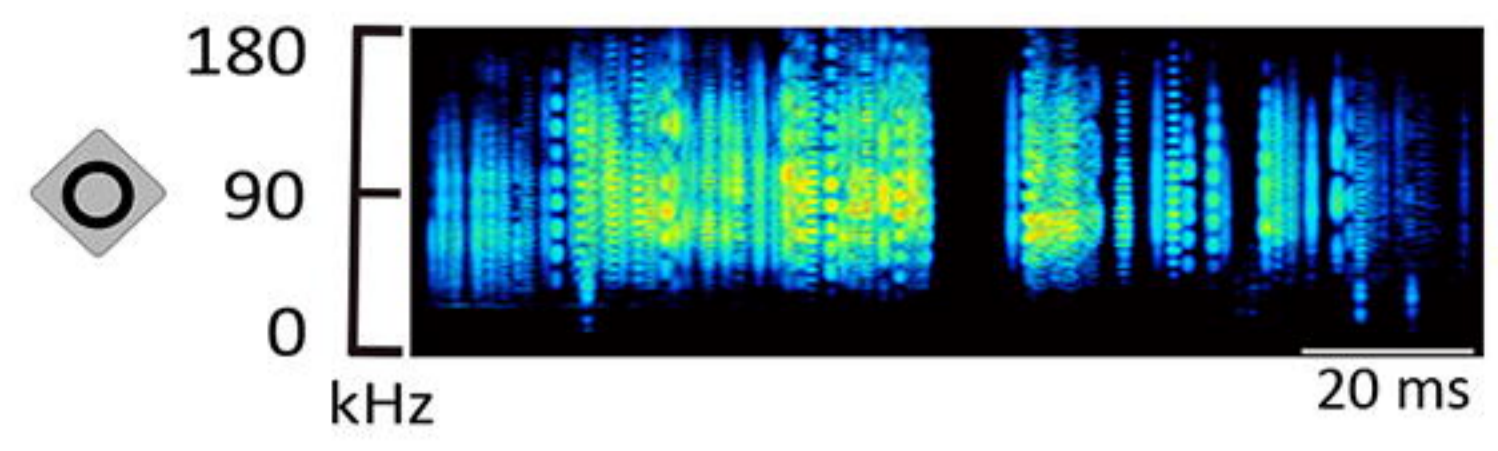
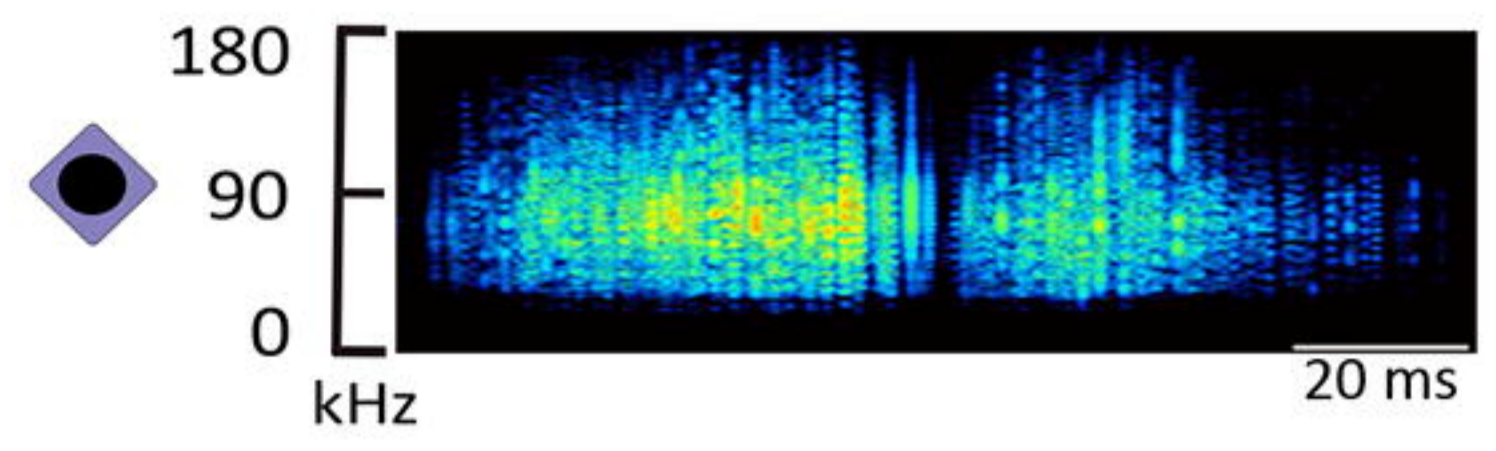
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- Macroglossinae
- Calpinae
- Acraginae
- Arctiinae
- Hypocalinae
- Ennominae



-85 dB -28 dB