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LETTERS

Multimodal warning signals for a multiple predator world

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Aposematism is an anti-predator defence, dependent on a predator's ability to associate unprofitable prey with a prey-borne signal[1]. Multimodal signals should vary in efficacy according to the sensory systems of different predators; however, until now, the impact of multiple predator classes on the evolution of these signals had not been investigated[2,3]. Here, using a community-level molecular phylogeny to generate phylogenetically independent contrasts, we show that warning signals of tiger moths vary according to the seasonal and daily activity patterns of birds and bats—predators with divergent sensory capacities. Many tiger moths advertise chemical defence[4,5] using conspicuous colouration and/or ultrasonic clicks[3,6]. During spring, when birds are active and bats less so, we found that tiger moths did not produce ultrasonic clicks. Throughout both spring and summer, tiger moths most active during the day were visually conspicuous. Those species emerging later in the season produced ultrasonic clicks; those that were most nocturnal were visually cryptic. Our results indicate that selective pressures from multiple predator classes have distinct roles in the evolution of multimodal warning displays now effective against a single predator class. We also suggest that the evolution of acoustic warning signals may lack the theoretical difficulties associated with the origination of conspicuous colouration.

Insectivorous birds and bats are major predators of adult Lepidoptera. In south-eastern Ontario, Canada, where the field data for this study were collected, residential and migratory insect-eating birds actively forage before, during and after the seasonal emergence of tiger moths[7–9]; a slight plateau in bird abundance occurs between early June and early July[7,8]. Conversely, peak bat foraging activity does not occur until early July and lasts until mid August. In early May, bat foraging activity is at ~15% of its peak, rising to only ~50% by late June[10–12]. Most insectivorous birds are diurnal predators sensitive to wavelengths extending beyond the human visual spectrum to the ultraviolet[13,14]. Vespertilionid bats are nocturnal, with their scotopic vision and poor visual acuity unsuited to the discrimination of insect prey[15]. Instead, these bats detect and locate prey and other objects in their immediate environment using the echoes returning from their mostly ultrasonic calls (>20 kHz). Birds do not echolocate prey nor are their ears sensitive to frequencies above 10 kHz (their range of best frequency is 2–5 kHz)[16]. However, both insect-eating birds and bats readily learn taste aversions to novel prey when prey cues are associated with toxicity[17,18]. This adaptive specialization of learning may be a necessary precondition in predators for the evolution of aposematic signalling in prey[17].

Many tiger moth species are unpalatable to birds and bats[3–6,15]. All species possess bat-detecting ears, and some respond to aerial hawking bats with ultrasonic clicks from sound-producing organs known as tymbals[6,10,15,19]. Many are also visually conspicuous[3,4,15,20], which may allow tiger moths to be more diurnal than most moths[20]. For each of the 26 species included in our study, colouration/pattern were scored as low contrast (cryptic; v1), white (conspicuous; v2) or high contrast (conspicuous; v3) (Fig. 1). Each species was also scored as

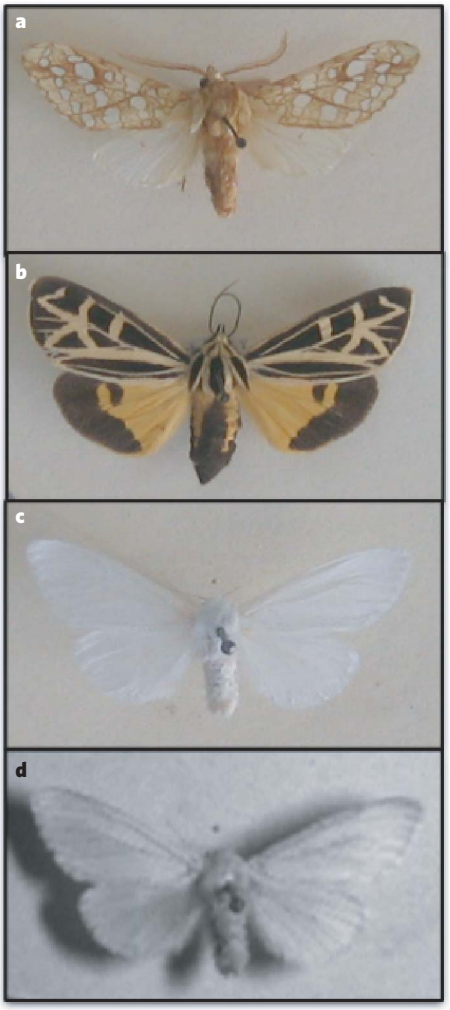

Figure 1 | Representatives of each visual class. a, Low colour contrast: *Lophocampa caryae*. **b**, High colour contrast: *Grammia anna*. **c**, White: *Hyphantria cunea*. **d**, Ultraviolet photograph of *H. cunea*, one of the 37 moth species scored as not exhibiting a qualitative difference in pattern between colour (**c**) and ultraviolet (**d**) photographs. Only *Lycomorpha pholus* was noted as having a different pattern under ultraviolet light (see Methods).

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being silent (a1), a simple-ultrasound producer (a2) or a complex-ultrasound producer (a3) (Fig. 2). The emergence date for each species was taken as the date of first capture at or in the vicinity of the study site[9]. Diel flight periodicity (DFP, 24-h activity pattern) for each species was determined using a previously reported behavioural assay[20] and percentage nocturnality was calculated using a site-specific rubric[20] (for details of trait quantification, see Methods Summary and Methods).

We built a community-level molecular phylogeny using one mitochondrial (*cytochrome oxidase I*, *COI*) locus and two nuclear (elongation factor 1a, *EF1a,* and *wingless*) loci (Fig. 3, see Methods Summary and Methods). For each species, character values for each of the four traits (emergence date, percentage nocturnality, visual class, acoustic class) were mapped onto this phylogeny (Fig. 3), which we then used to perform phylogenetically independent contrasts using Comparative Analysis of Independent Contrasts (CAIC)[21] version 2.6.9 (see Methods). Percentage nocturnality (hereafter, nocturnality) was not related to the emergence date ($F_{1,24} = 1.39$, $r^2 = 0.06$, $P = 0.25$). The emergence date (hereafter, emergence) was not related to the visual category (Fig. 4a, analysis of variance, ANOVA $F_{2,22} = 1.601$, $P = 0.209$). Nocturnality was significantly higher in low-contrast species than in white and high-contrast species, which did not differ significantly from one another (Fig. 4b, ANOVA $F_{2,22} = 9.546$, $P < 0.001$; Tukey HSD (honestly significant difference) post-hoc comparisons: low contrast versus white, $P = 0.001$; low contrast versus high contrast, $P = 0.001$; white versus high contrast, $P = 0.985$). Emergence occurred significantly later in the season for sound-producing species than for silent species (Fig. 4c, ANOVA $F_{2,22} = 5.593$, $P = 0.006$; Tukey HSD post-hoc comparisons: silent versus complex, $P = 0.005$; silent versus simple, $P = 0.059$; simple versus complex, $P = 0.63$). Nocturnality was not related to the acoustic category (Fig. 4d, ANOVA $F_{2,22} = 0.534$, $P = 0.588$). We used the program Mesquite version 2.0 (ref. 22) to investigate potential phylogenetic correlations between sound production and colouration considered as binary traits (silent (a1) or sound-producing (a2 + a3); low contrast (cryptic; v1) or white/high contrast (conspicuous; v2 + v3)) and found none ($P = 0.726$).

Differences in insectivorous bird and bat daily and seasonal activity patterns in the Nearctic allow for the use of DFP and emergence as proxies for species-specific differences in moths' exposure to these two predator classes. The correlation between reduced nocturnality and both classes of conspicuous colouration (Fig. 4b), and the lack of correlation between the latter and emergence (Fig. 4a), is as predicted by the relatively stable seasonal abundance of diurnal, vision-dependent insect-eating birds. Conversely, the positive correlation between emergence and sound production (Fig. 4c) is as predicted by the increasing selective pressure put on moths by nocturnal, echolocating bats as the season progresses[10]. The lack of correlation between nocturnality and sound production may be because, except for *Lycomorpha pholus*, all species are greater than 40% nocturnal and 19 out of 26 species are 60% or more (Fig. 3). The significant difference in emergence between silent and complex-sound-producing tiger moths corroborates evidence that, for bats, complex sounds are more salient warning signals than those directed at other sensory modalities[5,15,23]. More than this, the lack of positive correlation between sound production and conspicuous colouration argues against the evolution of multimodal warning signals for the function of improving learning and memory in either single predator class.

Warning signals function by informing would-be predators that the sender is unprofitable as prey[1–3]. The visual and acoustic warning signals of tiger moths have been shown to be readily associated with toxicity in birds and bats, respectively[3,5,23]. However, most visual aposematic signals are continuously displayed[2]; in short, they are easily seen. The fixation of an initially rare visually conspicuous phenotype is therefore paradoxical[2,3]. One possible solution is that predator avoidance of novel foods and reluctance to add these foods to the diet—behaviours common among birds[3]—may allow initially rare phenotypes to become more frequent in the population[2,3]. But because many bats are cavalier with respect to diet selection, attacking muted tiger

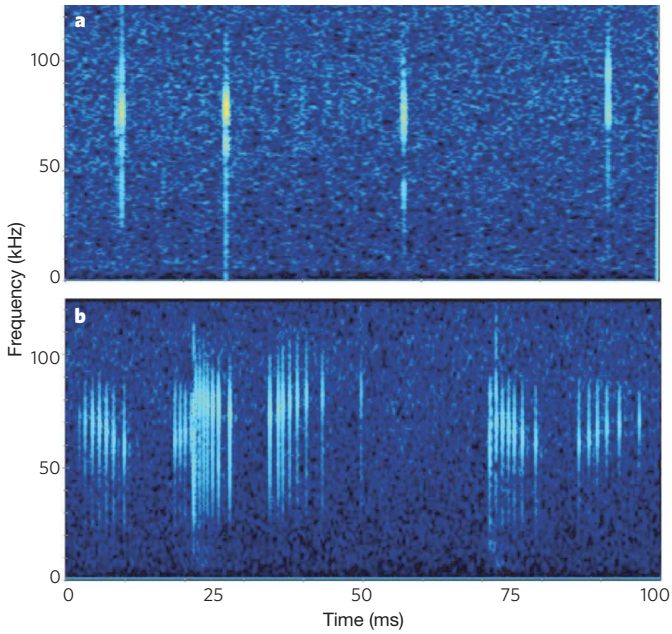

Figure 2 | Representative sonograms. a, Simple-ultrasound producers (for example, *Cisseps fulvicollis*). **b**, Complex-sound producers (for example, *Cycnia tenera*).

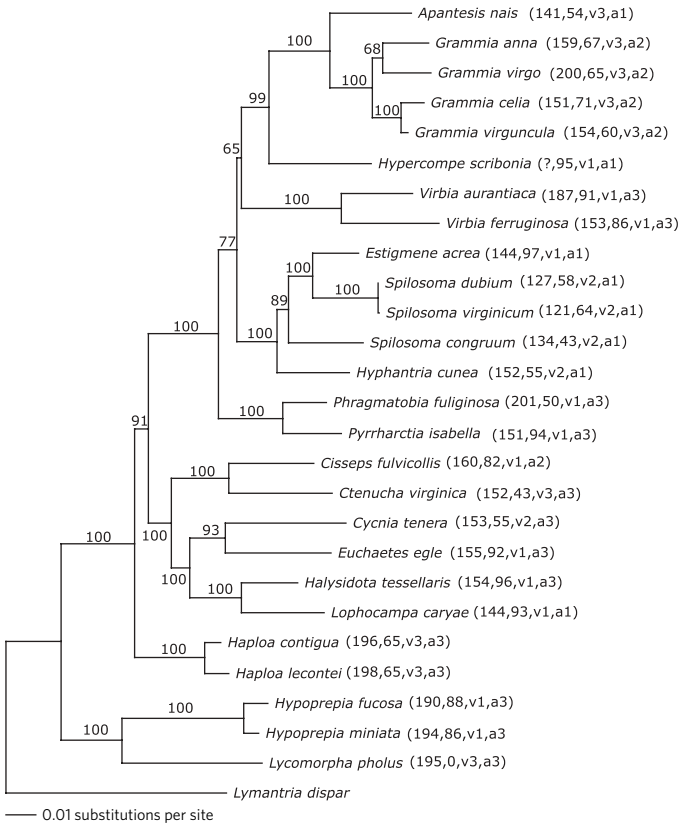

Figure 3 | 50% majority consensus phylogram of the bayesian trees. Numbers along the branches are bayesian posterior probabilities. Within parentheses after each species name are the character traits for that species: emergence (for example, 30 June 2007 = 181st day of the year), percentage nocturnality, visual class and acoustic class. All combinations of visual and acoustic signal classes were observed except for white, simple-sound-producing moths (v2,a2).

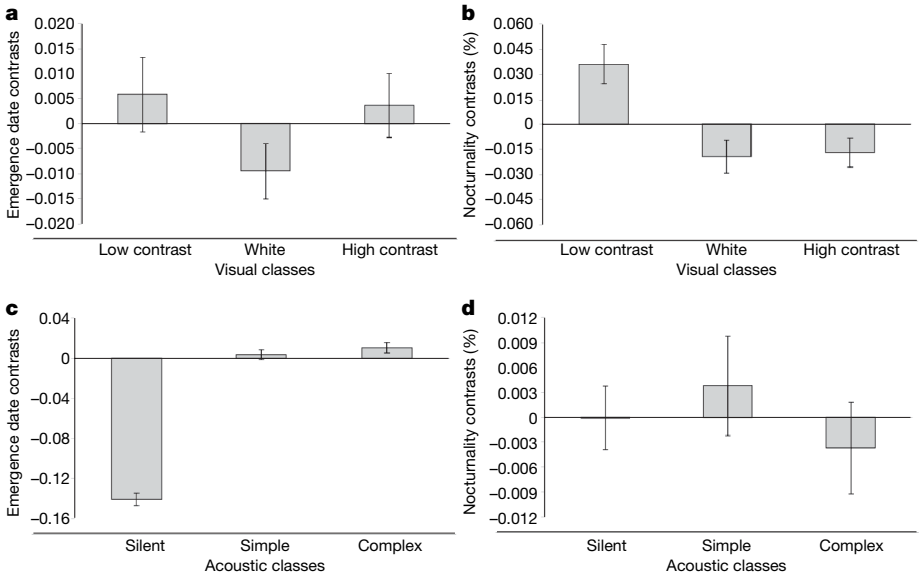

Figure 4 | Phylogenetically independent contrasts (mean ± s.e.). **a**, Emergence date contrasts (calculated in CAIC v. 2.6.9) in relation to three visual classes (low colour contrast, white, high colour contrast). **b**, Percentage nocturnality contrasts in relation to three visual classes. **c**, Emergence date contrasts in relation to three acoustic classes (silent, simple-sound producers, complex-sound producers). **d**, Percentage nocturnality contrasts in relation to three acoustic classes.

moths and moth-sized inanimate objects under otherwise natural conditions[24,25], the ability of rare phenotypes to increase due to predators' wariness[2,3] may not apply during bat–moth interaction. However, unlike visual signals, during these interactions the ultrasonic clicks of tiger moths are elicited only by the echolocation calls of, or contact with, an attacking bat[15,19,26]. These signals are thus 'invisible' to bats at a distance and are produced by a tiger moth only after a bat has already detected it and is on course for interception; they therefore may be exempt from the rare/conspicuous paradox.

Our study is one of few to investigate the evolution of aposematic signalling using a phylogenetic framework[2,3], and is the first, to our knowledge, to consider the evolution of multimodal displays selected by multiple predators. Multiple sensory signals have been suggested to act synergistically to reinforce aversion learning[3,27], and the visual and acoustic aposematic signals of tiger moths and other protected prey have been suggested to have evolved to serve a single, synergistic function[27]. Indeed, sounds audible to chicks, *Gallus gallus domesticus*, can improve visual discrimination learning[3,28], and the lower periphery of the frequencies found in some tiger moth clicks may be audible to birds at very close range (that is, when held in the bird's beak). However, in our system only two sound-producing moths are >50% diurnal (Fig. 3). At least in the case of tiger moths, this dearth of diurnal sound producers provides evidence against multiple signals having initially evolved in response to selective pressures from a single predator class and/or that, once evolved, they are maintained by selective pressures from a single predator class. Taken together, our results suggest that the proximate benefits of some multimodal displays are not reflective of their evolutionary histories. These histories may be better understood in the context of selective pressures from multiple predator classes—classes defined by their own sensory capacities and life history traits.

METHODS SUMMARY

All bat, bird and moth activity and emergence data were collected at or near Queen's University Biological Station in south-eastern Ontario, Canada[6–12,20]. For DFP, we used previously reported data[20] for 10 out of the 26 tiger moth species, and used the same setup and design to collect data for the remaining 16 species ($N = 4$ per species). For acoustic classification, data for all but three species were taken from the literature[6,10,15,19,23,24,26]. Species that produced sounds at a maximum rate of <100 clicks per s were classified as 'simple', and those that produced sounds at a maximum rate of >500 clicks per s were classified as 'complex'. No species had a maximum rate between 100 and 500 sounds per s. *Hypercompe scribonia* did not produce sounds and was scored as 'silent'. Sounds produced by *Cisseps fulvicollis* and *Ctenucha virginica* were recorded and analysed as described elsewhere[15]. For visual classification, digital colour and ultraviolet photographs (custom setup described elsewhere[29]) of spread specimens of the 26 tiger moth species and 12 other sympatric noctuoids were analysed and classified using a computer-driven routine (see Methods). We asked human subjects ($N = 15$) to compare colour images to ultraviolet images to determine whether qualitative pattern changes exist between these sets of images (Fig. 1c, d). For phylogenetic inference, portions of one mitochondrial (*COI*) and two nuclear (*EF1a* and *wingless*) genes totalling ~2 kilobases were amplified and sequenced (see Methods). The topology and branch lengths of a 50% majority consensus phylogenetic tree constructed using MrBayes 3.1.2 (ref. 30) were used to calculate standardized linear contrasts using the program CAIC[21]. All comparative analyses reported herein use actual branch lengths.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions M.L.N. was responsible for genetic analyses. J.M.R. was responsible for behavioural, comparative and signal analyses. J.M.R. wrote the manuscript.

Author Information All sequences were submitted to GenBank under accession numbers EU333575–EU333652. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.L.N. (mln32@cornell.edu) or J.M.R. (jmr@biology.sdu.dk).

METHODS

Colour quantification and visual categorization. Digital colour photographs of the 26 tiger moth species used in this study and of 12 sympatric noctuoid species were taken outdoors under full sunlight using specimens from the Cornell University Insect Collection. We used a D40x 10.2 MP digital SLR camera with a 18–55 mm f/3/5-5.6G ED II AF-S DX Zoom-Nikkor Lens (Nikon Inc.). White balance was set manually using a Zebra 2-sided grey card (Novoflex). Shutter speed was adjusted for optimal exposure within a range of 1/125 s–1/500 s for each photograph while the aperture was set to F8 for the entire session. Each colour photograph was calibrated according to previously described methods[31], using the six greys of the Gretag Macbeth Mini ColourChecker Chart (Gretag Macbeth AG/LLC), which was included in each moth photograph. All photographs were also calibrated globally to account for changes in sunlight during the photography session (that is, for colour photographs, Macbeth white was set equivalent to red (R) = green (G) = black (B) = 255; black was set equivalent to R = G = B = 0). TIFF files were exported to Image J v. 1.38x (National Institutes of Health) from which we saved *x,y* coordinates from the perimeter of each moth. DigitalColour Meter v. 3.4.1 (Apple Computer) was used to measure 8-bit (256-point) corrected RGB values for ten points for each species; sampled points were determined using the intersection of two randomly selected and matched vectors (each taken from randomly selected pairs of *x,y* coordinates). A single reflectance value was taken for each of the ten homologous points for each moth from the ultraviolet photographs. Each ultraviolet photograph was calibrated using Macbeth black (set equivalent to R = G = B = 0) and a Spectralon (Labsphere) white reflectance standard (set equivalent to R = G = B = 255); the chart and standard were included in each photograph. We input the four values (R, G, B, ultraviolet) for each of the ten points sampled for each species in the cluster analyses outlined below.

White moths were classified as such and were removed from further colour analysis for three reasons: first, white is conspicuous on many natural backgrounds; second, none exhibited pattern change under ultraviolet illumination (see Fig. 1c, d); and third, it is unclear whether white Lepidoptera possess warning colouration[3]. In JMP v. 7 (SAS Institute), we used hierarchical clustering (centroid, data not standardized) and derived distance values based on RGB/ultraviolet data for the remaining 33 species; using this same method, we input species distance values and found two clusters: cluster A (species of <170 distance units: all 'low contrast/cryptic' tiger and all 12 other noctuoid species) and cluster B (species >200 distance units: all 'high contrast/conspicuous' tiger moth species). None of the moths in cluster B (high contrast/conspicuous) had patterns indicative of disruptive colouration[3].

Phylogenetic inference and comparative analyses. Genomic DNA of one individual of each of the 26 ingroup species and of 1 outgroup species (*Lymantria dispar*) was extracted from the flight muscle tissue of whole moths stored in 95% ethanol using a Qiagen DNeasy Tissue Kit. 764 bp from mitochondrial *COI* and tRNA-leucine were amplified using the primers C1-J-2183 (alias Jerry), TL2-N-3014 (alias Pat), C1-J-2195 (alias CO1RLR) and TL2-N-3014 (ref. 32). 845 bp from *EF1a* were amplified using the primer pairs M44-1/rcM53-2, rcm4/M52.7 and ef44/rcM52.6 (refs 33, 34). We also used an internal arctiid-specific primer pair developed from an alignment of a subset of the 26 species: Internal Forward (5′ ACGTTCTTTACGTTGAAACCAAC 3′)/Internal Reverse (5′ GGACACAGAGATTTCATRAAGAACAT 3′) and a noctuid-specific primer pair developed from a consensus alignment of 18 noctuid species sequenced in ref. 35: Noctuidae Forward (5′ TTCGAGAAGGARGCCCAG 3′)/Noctuidae Reverse (5′ GAGGGAAYTCYTGGAAGGA 3′). We were only able to obtain a portion of the 845 bp sequence for *C. tenera* and *Euchaetes egle*, so these species were excluded from the final alignment for this locus. 457 bp from *wingless* were amplified using the published primer pairs LepWG1/LepWG2 and LepWG2/LepWG2a[36]. We also designed internal arctiid-specific primers using the alignment of several of the 26 species: WGIntF (5′ TGGTCTGGATTATGAGG CCGCA 3′) paired with LepWG1 and WGIntR (5′ TCTGGCTCGTGC ACGGTTAAGACC 3′) paired with LepWG2. We were unable to amplify *E. egle* for this locus, so this species was not included in the final *wingless* alignment. *L. dispar* was selected as the outgroup for this analysis because this species is closely related to the arctiids[35] and has been used as the outgroup in previous arctiid phylogenies[37].

PCR amplification was performed using conditions available from the authors. PCR products were cleaned using Exonuclease I and Shrimp Antarctic Phosphatase, and then purified on Sephadex columns (Sigma-Aldrich). The purified products were sequenced with a Big Dye Terminator Cycle sequencing kit and an ABI-3100 automated sequencer (Applied Biosystems) with the same primers used for amplification. The program Aligner (CodonCode Corporation) was used to edit and align the sequences.

MODELTEST 3.7 (ref. 38) was used to determine the best-fit model of nucleotide substitution for each locus. Using an AIC (Akaike information criterion) approach, which measures the fit of various nucleotide substitution models to the data, the best-fit model was GTR + I + G (where GTR is General Time Reversible, I is the proportion of invariable sites, and G is the shape parameter of the gamma distribution) for mitochondrial *COI*, SYM + I + G for *EF1a* and *wingless*, and GTR + I + G for the three loci combined. The GTR + I + G model was used in the combined maximum likelihood analysis of the three loci in PAUP* 4.0 (ref. 39). The tree resulting from this analysis (not shown) has a nearly identical topology to the 50% majority consensus tree from the bayesian analysis.

MrBayes 3.1.2 allows the user to apply the best-fit model of nucleotide substitution to each locus separately in a combined analysis. As determined from MODELTEST 3.7 above, the GTR + I + G model was applied to the mitochondrial *COI* locus and the SYM + I + G model to the *EF1a* and *wingless* loci. The analysis ran for 10-million generations, with sampling every 1,000 generations. The average value of the potential scale reduction factors was 1.00 and the average standard deviation of split frequencies at the end of the run was 0.001, demonstrating convergence. The first 2,000 trees were eliminated as burn-in, and a 50% majority-rule consensus tree was created using PAUP*4.0. The MrBayes runs were performed at Cornell's Computational Biology Service Unit.

For the comparative analysis using this bayesian tree, we used phylogenetically transformed dummy variables to allow for statistical analyses using both continuous (emergence date and percentage nocturnality) and discontinuous categorical (visual and acoustic signals) traits[40].

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