

## ECOLOGY

## Fireflies thwart bat attack with multisensory warnings

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Many defended animals prevent attacks by displaying warning signals that are highly conspicuous to their predators. We hypothesized that bioluminescing fireflies, widely known for their vibrant courtship signals, also advertise their noxiousness to echolocating bats. To test this postulate, we pit naïve big brown bats (*Eptesicus fuscus*) against chemically defended fireflies (*Photinus pyralis*) to examine whether and how these beetles transmit salient warnings to bats. We demonstrate that these nocturnal predators learn to avoid noxious fireflies using either vision or echolocation and that bats learn faster when integrating information from both sensory streams—providing fundamental evidence that multisensory integration increases the efficacy of warning signals in a natural predator-prey system. Our findings add support for a warning signal origin of firefly bioluminescence and suggest that bat predation may have driven evolution of firefly bioluminescence.

## INTRODUCTION

Pronounced advertisement of identity and location is a common strategy of defended animals (1). These brazen prey warn predators of the cost of attack with clear and salient signals. Vivid poison dart frogs warn visually specialized predators of toxins stockpiled in their skin glands (2, 3), sea slugs reek odors to alert olfactory-specialist predators of stinging nematocysts (4), and tiger moths produce sonar-triggered bursts of ultrasound to warn echolocating bats of noxious taste (5, 6). Given the demonstrated efficacy of unisensory warnings, why do defended animals routinely integrate multiple sensory channels in their displays? A recent framework of functional hypotheses, adapted from work on complex communication signals (7), suggests many ways in which multisensory warning signals may be advantageous (8). Experimental evidence from artificial and seminatural paradigms points to several functions, such as enhanced avoidance learning (9). Yet, demonstration of these advantages in natural predator-prey systems is lacking, despite their potentially profound implications for the tempo and mode of speciation and extinction (10).

Here, we present evidence that toxic, bioluminescent fireflies (Lampyridae) transmit multisensory warning signals to echolocating bats. Predators across taxa show intense aversions to these noxious beetles (11–17), some dying within an hour of ingestion, likely from poisonous steroidal pyrones (lucibufagins) (18). Previous work (15, 19) and our own palatability experiments indicate that *Photinus* spp. fireflies are markedly distasteful to bats ( $n = 3$  bats; 7 fireflies, complete rejection; see movie S1 for strong aversive behaviors such as rapid head shaking and coughing). It is then unsurprising that fireflies are almost entirely absent from bat diets (15, 20) [the single documented exception (21) is disputed because of methodological issues (22)], despite abundant opportunities for predator and prey to interact (15, 22). Yet, it is unknown whether and how bats discriminate profitable prey from firefly foe.

## RESULTS AND DISCUSSION

To first test our hypothesis that fireflies warn bats of their chemical defense, we pit free-flying, bioluminescing fireflies (*Photinus pyralis*)

against three naïve big brown bats (*Eptesicus fuscus*) in a dark, anechoic flight room for 1 to 4 days and filmed their interactions using three high-speed cameras. Bayesian mixed-effects logistic regression of bat capture behavior demonstrates strong support for learned avoidance of these fireflies [intact  $\beta$  – control  $\beta = -4.691$ ; 95% credible interval (CI),  $-8.53$  to  $-1.969$ ; Fig. 1 and table S1]. All bats captured at least one firefly on the first night of presentation (range, 1 to 3; three to four fireflies presented per night, comprising 25% of total prey for all experiments) and subsequently dropped the beetle. Over just a few nights, the bats in our experiment learned to avoid these chemically protected animals (movie S2). Control palatable, nonbioluminescent scarab beetles, and pyralid moths (75% presentation rate throughout) pit against bats during the same foraging nights were consistently captured (98.7% capture success;  $n = 242$ ; Fig. 1A). Since at least the 19th century, naturalists have hypothesized that fireflies warn bats of their “offensiveness” (23, 24), and here, we provide definitive evidence.

As an additional test, we pit two bats that learned to avoid fireflies against “darkened” fireflies that were unable to produce light for another one to two nights. We thoroughly painted firefly bioluminescing organs, including adjacent abdominal segments, to block all light production. One bat captured all darkened fireflies ( $n = 2$ ) and controls ( $n = 2$ ), demonstrating that bioluminescence was used for avoidance learning by this bat. This observation is in line with mounting evidence that bats integrate echolocation with vision when foraging (25, 26) and that insectivorous bat retinas contain photoreceptors sensitive to the dominant wavelengths of firefly bioluminescence (27, 28). A second bat avoided all darkened fireflies ( $n = 3$ ) and captured all controls ( $n = 6$ ), indicating that another source of information was sufficient for this bat to discriminate noxious fireflies.

To test whether bats can learn to discriminate fireflies without bioluminescence, we pit darkened fireflies against four additional naïve big brown bats for 1 to 4 days. These bats learned to avoid darkened fireflies (darkened  $\beta$  – control  $\beta = -2.306$ ; 95% CI,  $-5.783$  to  $-0.302$ ; Fig. 1), although they learned at a slower rate and to a lesser degree than those pit against free-flying, bioluminescing fireflies (intact  $\beta$  – darkened  $\beta = -2.385$ ; 95% CI,  $-5.222$  to  $-0.023$ ; Fig. 1). How were bats able to differentiate control prey from darkened fireflies? Olfactory information is a possibility. However, here and in three other studies (15, 17, 19) that performed palatability tests by presenting nonflying bats with chemically defended fireflies, these predators showed no ability to use smell to reject these noxious beetles, instead waiting to reject until tasting the prey. Determining whether bats use volatile components of firefly chemistry to avoid fireflies in short-duration, high-speed aerial interactions

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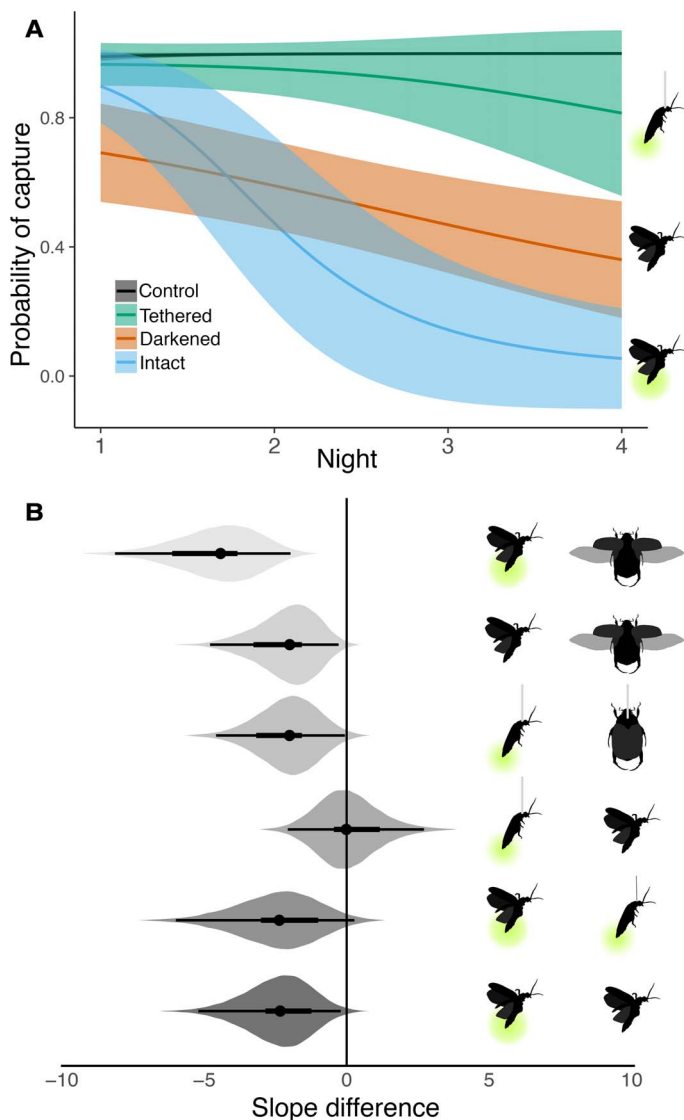
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**Fig. 1. Bats learn to avoid unisensory and multisensory firefly warnings.** (A) Probability that bats capture firefly treatments and control prey over successive nights. Line, mean; shading, 95% CI. (B) Comparisons of avoidance learning between prey types (depicted as silhouettes on right; scarab beetle silhouette represents pooled scarab beetle and pyralid moth control data). Bats learn to avoid all firefly treatments. Avoidance learning is defined here as a slope less than zero in comparisons of firefly treatments and scarab controls. For comparisons of firefly treatments, negative values indicate faster avoidance learning of prey shown on left. There is no difference in learning rates between unisensory (tethered versus darkened) treatments. Intact fireflies emitting multisensory warnings induce faster avoidance learning compared to either unisensory treatment. Circle, median; thick line, 68% CI (which is  $\pm 1$  SD under a normal distribution); thin line, 95% CI.

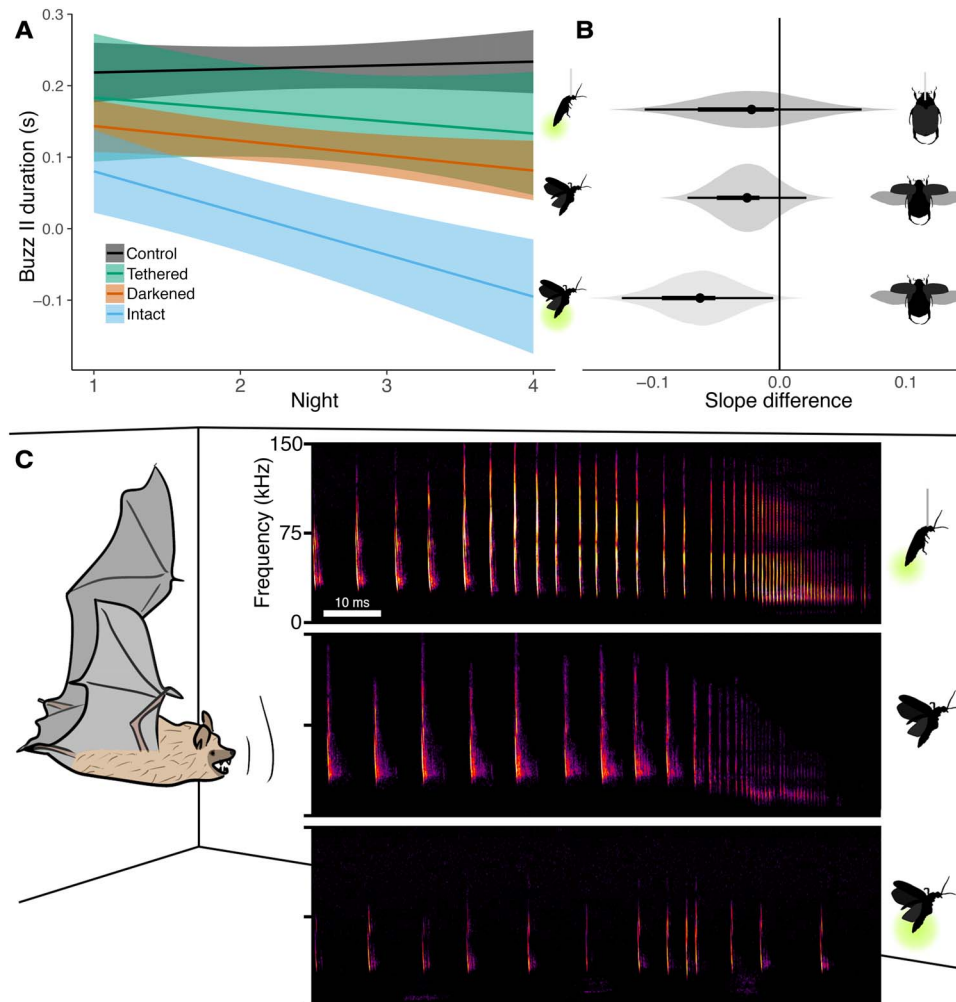
requires additional work. Insectivorous bats have some of the smallest olfactory organs in mammals and invest significantly less in olfactory tissues than bats with frugivorous or mixed (frugivorous and insectivorous) diets (29). Understanding the role of olfaction in bat-insect interactions is an important frontier.

Alternatively, bats may have learned to avoid darkened fireflies by extracting acoustic information. Bats might use echo-derived shape, size, and texture (30, 31), the amplitude and spectral modulation im-

posed by wingbeat rate on the returning echo stream (32, 33), or overall three-dimensional flight paths to associate fireflies with noxiousness. Fireflies fly with the nonchalance of a chemically protected insect in a slow, nonerratic flight path. Poulton (34) reported this type of movement behavior in 1890 to accompany visual warning signals in diurnal moths, and it has since been reported in chemically protected butterflies (35) and nocturnal tiger moths (36). Previous laboratory work with a rotating device mimicking a fluttering insect has shown that big brown bats can discriminate the different wingbeat rates in the range of the insects we presented to bats here (mean  $\pm$  SE; firefly,  $63.3 \pm 1.9$  Hz,  $n = 4$ ; scarab,  $93.3 \pm 1.9$  Hz,  $n = 4$ ; pyralid moth,  $44.3 \pm 1.8$  Hz,  $n = 4$ ; see Materials and Methods) (37, 38). Wingbeat rates have been implicated as a component of warning signals in systems with visually guided predators. Convergence in wingbeat rates within aposematic mimicry groups occurs in heliconiine butterflies (39, 40), as well as in damselfly mimics of ithomiine butterflies (41). Considering the prevalence of chemically protected flying insects (8), the roles of flight-based warning signals, transduced by vision, echolocation, or passive listening are fertile grounds for future research, particularly as it applies to multisensory signaling.

To further parse the roles of bioluminescent and echoic information in avoidance learning, we presented immobile fireflies to one bat on a monofilament tether, rendering the beetles flightless but preserving their ability to produce their bioluminescent displays. We gently pushed these tethered prey into a swinging motion to stimulate light production. As a control for this experiment, we also presented size-matched, flightless pyralid moths and scarab beetles in the same pendulum motion on the tether. The bat learned to avoid these fireflies (tethered  $\beta -$  control  $\beta = -2.085$ ; 95% CI,  $-4.412$  to  $-0.088$ ; Fig. 1), although to a lesser degree than free-flying, bioluminescing fireflies (intact  $\beta -$  tethered  $\beta = -2.607$ ; 95% CI,  $-6.333$  to  $0.228$ ; 96.2% probability that difference between treatments is not 0; Fig. 1B) but comparable to free-flying, darkened fireflies (tethered  $\beta -$  darkened  $\beta = 0.222$ ; 95% CI,  $-2.006$  to  $3.534$ ; Fig. 1B). Thus, although unisensory firefly displays produced avoidance learning, the addition of flight or bioluminescence to either unisensory display potentiated this effect. To our knowledge, this is the first example of a multisensory warning display enhancing avoidance learning in a natural predator-prey system.

Using echolocation as a window into real-time information processing, we also investigated sonar behavior by bats during these experiments. Moments before a capture attempt, big brown bats actively update their acoustic scenes by emitting sonar cries at a rapid rate. The first part of this “buzz” behavior (buzz I) gives way to buzz II just before capture, wherein rapid cry rate continues, but the fundamental frequency of the cries is lowered by an octave. Both high call rate and lowered frequency are putative adaptations for tracking moving prey from short distances (42). Here, bats shortened their buzz II durations as they gained experience with free-flying, bioluminescing fireflies (intact  $\beta -$  control  $\beta = -0.063$ ; 95% CI,  $-0.125$  to  $-0.004$ ; Fig. 2, A and B, and table S2). During nights 3 and 4, bats often did not buzz these fireflies (Fig. 2C). Bats facing darkened or tethered fireflies did not reduce buzz II duration over time and continued extracting additional information from these prey throughout our experiments (darkened  $\beta -$  control  $\beta = -0.025$ ; 95% CI,  $-0.073$  to  $0.022$ ; tethered  $\beta -$  control  $\beta = -0.021$ ; 95% CI,  $-0.106$  to  $0.065$ ; Fig. 2, A to C). In addition, during interactions with all prey, bats did not alter their buzz I durations (fig. S1 and table S3). These sonar data reinforce our behavioral findings that prey signaling through multiple senses enhances predator discrimination learning in this system.



**Fig. 2. Bat sonar behavior.** (A) The duration of bats' final echolocation phase (buzz II) presented against successive nights of interactions with different firefly treatments. Line, mean; shading, 95% CI. (B) Between-prey comparisons of changes in buzz II durations. Lower values indicate greater reduction of buzz duration over time for prey on left. Compared with the control, bats reduced their buzz II duration as they learned to avoid intact fireflies. Interactions with darkened or tethered fireflies did not affect buzz II durations. (C) Example spectrograms of echolocation behavior during the last night of interactions with each firefly treatment. Bats entered buzz II during interactions with tethered and darkened fireflies, but not for interactions with intact firefly. Each spectrogram depicts the final 1 s of an interaction. Pseudocoloration illustrates intensity gradients within a single spectrogram but is not standardized across spectrograms.

The data we present here triangulate onto a clear aposematic signaling relationship between bats and fireflies. Past studies and anecdotal observations have described bats that are sometimes attracted to artificial firefly-like lures and even attack fireflies (15, 43). In light of our findings, we contend that these data may reflect (i) observations of juvenile, inexperienced bats; (ii) artificial lures that do not accurately recreate the echoic and visual properties of real fireflies and thus initiate bat exploratory behavior; or perhaps, (iii) bat species that differ in their sensory abilities (44) or tolerance of firefly chemical defenses (6).

Our data also reveal intriguing aspects of bat sensory biology. For bats with a frequency-modulated sonar strategy, such as the big brown bats studied here, the use of echo-derived information in an ecologically relevant prey discrimination task is a novel observation that expands our understanding of bat perception. Further, we provide evidence that visual aposematic signals are sufficient for transmitting warnings of chemical defense to bats, opening up the possibility that other protected insects (for example, tiger moths) use this sensory channel to communicate with these nocturnal predators. Our experiments provide empirical support

for several functional hypotheses developed for multisensory warnings (8). Regarding signal content, bioluminescence and echoic components separately generated avoidance learning, indicating that they likely provide redundant information of prey defense to bats. The deterrence capability of individual sensory components may be particularly important for firefly survival if bats interact with fireflies between flashes [which are 0.4 to 8.0 s apart for *Photinus* spp.; (45, 46)] or if the light environment reduces the flash's contrast against the background. These redundant unisensory components may also be separately targeting not only distinct taxa with divergent sensory systems [for example, (47)] but also intraspecific perceptual variation within bats, as we have documented here (7). It is clear that the integration of visual and echoic signal components by fireflies enhances avoidance learning by bats, resulting in learning rates comparable to those elicited by ultrasonically advertising, distasteful tiger moths (5, 6).

The evolutionary implications of our data are nontrivial. Larval bioluminescence is a shared, derived characteristic of all fireflies, and there is experimental (12, 14) evidence that these "glow-worms" advertise chemical defenses to predators. In addition, current phylogenies

support the hypothesis that adults of the ancestral firefly lineage did not use bioluminescence in sexual communication, instead relying on pheromones for mating (48, 49). Most fireflies that use only pheromones for sexual communication are diurnal (48). It follows that as ancestral lineages of fireflies shifted to nocturnal activity, they experienced heavy selective pressure from bats and likely other predators [for example, spiders (16)], which drove the evolution of adult bioluminescence. We postulate that each time bioluminescence has evolved [at least six times in fireflies (49) with at least two additional origins in nonfirefly beetles], selective pressure to broadcast warnings of chemical defense initially shaped the signal, which then was secondarily co-opted for mating. A recent dating analysis of Coleoptera (50) places the origin of fireflies at ~75 million years ago (MYA), just before the emergence of bats ~65 MYA (51). For fireflies on the wing in the night sky, the predators to avoid have been, and continue to be, bats. We predict that a time-calibrated firefly phylogeny will reveal that bioluminescence emerged with bats and that bats may have invented fireflies.

## MATERIALS AND METHODS

### Behavioral experiments

We mist-netted eight big brown bats (*E. fuscus*) in southwest Idaho under Idaho Department of Fish and Game permit #110615. All bats were housed and cared for at Boise State University following the Institutional Animal Care and Use Committee protocol #006-AC14-014 and care protocols established by Lollar and Schmidt-French (52). The light regime was 10-hour dark/14-hour light. Before behavioral trials, we trained bats to hunt greater wax moths (*Galleria mellonella*) suspended from a monofilament tether. Wild, male fireflies (*P. pyralis*) were collected in Lehigh County, PA and Douglas County, KS, while control scarab beetles (*Callistethus marginatus* and *Dyscinetus morator*) were collected in Alachua County, FL in June to July 2015. Big brown bats occur throughout North America and are considered “nonmigratory” (53), while fireflies that flash as adults are generally only found east of the Rocky Mountains. We thus assumed that the bats used in these experiments were naïve to flashing, noxious fireflies.

We conducted bat-firefly behavioral experiments in a sound-attenuated flight room (6.8 × 5.6 × 3.9 m) illuminated by Wildlife Engineering infrared light-emitting diode arrays and red-filtered lighting. To record bat-prey interactions, we captured streaming video from three high-speed, infrared-sensitive cameras [Basler scout, 100 frames per second (fps)] using a National Instruments PCIe-8235 GigE Vision frame grabber and a customized LabVIEW program. Simultaneously, we recorded echolocation behavior using four ultrasonic Avisoft microphones [three CM16, ±3 dB(Z), 20 to 140 kHz; one USG Electret Ultrasound Microphone, ±9 dB(Z), 20 to 120 kHz] connected to a four-channel Avisoft UltraSoundGate 416H (sampling at 300 kHz) via XLR cables and recording to a desktop computer running Avisoft-RECORDER software. We mounted microphones on the ceiling in the center of the room, placing each CM16 microphone 1.5 m apart from and encircling the central USG Electret mic. For all interactions, we synchronized audio and video recordings by triggering both with a National Instruments 9402 digital I/O module.

We presented *P. pyralis* fireflies to bats in one of three treatments: (i) intact, free-flying, and flashing ( $n = 3$  bats); (ii) intact, free-flying, and nonflashing ( $n = 4$  bats); and (iii) intact, tethered, and flashing ( $n = 1$  bat). To occlude bioluminescence for treatment #2, we painted all bioluminescent abdominal terga and adjacent terga, black or red (Testors Enamel Paints “GI Black” and “Red Cherry,” respectively). Both colored

paints effectively blocked bioluminescent transmittance. We also presented painted scarabs ( $n = 2$ ) to bats to control for the effect of paint. Once it was apparent that painted scarabs were caught and ingested with vigor, we then presented unpainted scarabs to minimize bat exposure to the enamel paints. Flight controls were wax moths, scarab beetles, or a combination of the two. Free-flying prey were hand-released, while tethered insects were suspended from an ~75-cm-long monofilament line attached to the ceiling. To avoid contaminating control prey, experimenters’ hands and the tether were washed following contact with each firefly. A trial began once we released the prey and we allowed bats to hunt for 1 min after release. We assessed capture behavior during experiments and confirmed captures by reviewing recordings of all interactions from all three cameras.

### Echolocation analysis

We analyzed audio files using Avisoft-SASLab Pro software. We inspected all four audio channels, beginning 900 ms before the selected interaction, and analyzed the channel with the highest signal-to-noise ratio [following (54)]. Since free-flight interactions occurred at unpredictable locations around the room, recordings of bats echolocating far or off-axis from the microphones resulted in low signal-to-noise ratios and were thus discarded from the analysis. When all echolocation calls of an interaction were distinct from background noise, we manually extracted buzz I and buzz II durations from the oscillogram. We did not assess frequency components of the calls, as the variable positioning of the bats in relation to the microphones would likely lead to inaccurate frequency data due to the effects of off-axis directionality and atmospheric attenuation.

### Wingbeat rates

To determine prey wingbeat rates, we manually counted the number of wingbeats over a 0.1- to 0.5-s interval using high-speed video recordings of flying prey (Edgertonic, monochrome, 500 fps, 1920 × 1080 resolution).

### Palatability experiment

We assessed the palatability of *P. pyralis* to three bats ( $n = 7$  total interactions) along with scarab and wax moth controls ( $n = 8$  total interactions; movie S1). To avoid contaminating control insects, we presented prey to each bat with forceps, which we washed following contact with fireflies. We scored palatability from 0 to 100% in accordance with methods outlined by Hristov and Conner (55). We added the following scores based on how much of the prey was consumed: head, 1; thorax, 2; abdomen, 3. For each proffered insect, the summed score was then divided by 6 and multiplied by 100 to obtain the palatability percentage.

### Statistical analyses

We used generalized linear mixed models fit under a Bayesian framework to examine differences between slopes of treatment groups in relation to night of the experiment (56, 57). To determine differences between slopes of treatments, we used models including fixed main effects of treatment and night of experiment and their interaction. We subtracted samples from posterior distributions of slopes of each treatment to obtain posterior distributions of differences between slopes of treatments. We determined differences when 95% CIs from the resulting distribution did not intersect zero. For further inference, we also examined the probability that a given parameter was different from 0. We pooled data from all controls (scarab beetles and pyralid moths) for the behavioral analysis and used scarab beetles for the control in the echolocation analysis.

All models included random intercepts and slopes for individual bats. We used a binomial distribution and logit link for modeling capture rate and a normal distribution and identity link for modeling duration of echolocation phases (buzz I and buzz II). We implemented the model in JAGS (Just Another Gibbs Sampler) version 4.2.0 (58) using the jagsUI package version 1.4.4 (59) and R version 3.2.3 (60). We ran three chains for 50,000 iterations after 10,000 burn-ins and a thinning rate of 2, yielding 60,000 draws from the joint posterior. We used standard weakly informative priors (57) and visually assessed traceplots and used the Gelman-Rubin statistic (61) to check for convergence. All models had good mixing of chains and  $\hat{R} < 1.1$  for all parameters.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/4/8/eaat6601/DC1>

Fig. S1. Comparisons of the change in buzz I duration between firefly treatments and control scarab beetles.

Movie S1. Firefly palatability.

Movie S2. Firefly avoidance.

Table S1. Change in probability of capture over time for each prey type and between-prey differences of these changes.

Table S2. Change in duration of buzz II over time for each prey type and between-prey differences of these changes.

Table S3. Change in duration of buzz I over time for each prey type and between-prey differences of these changes.

Data S1. Raw data of bat capture and echolocation behavior; prey palatability and wingbeat rate.

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