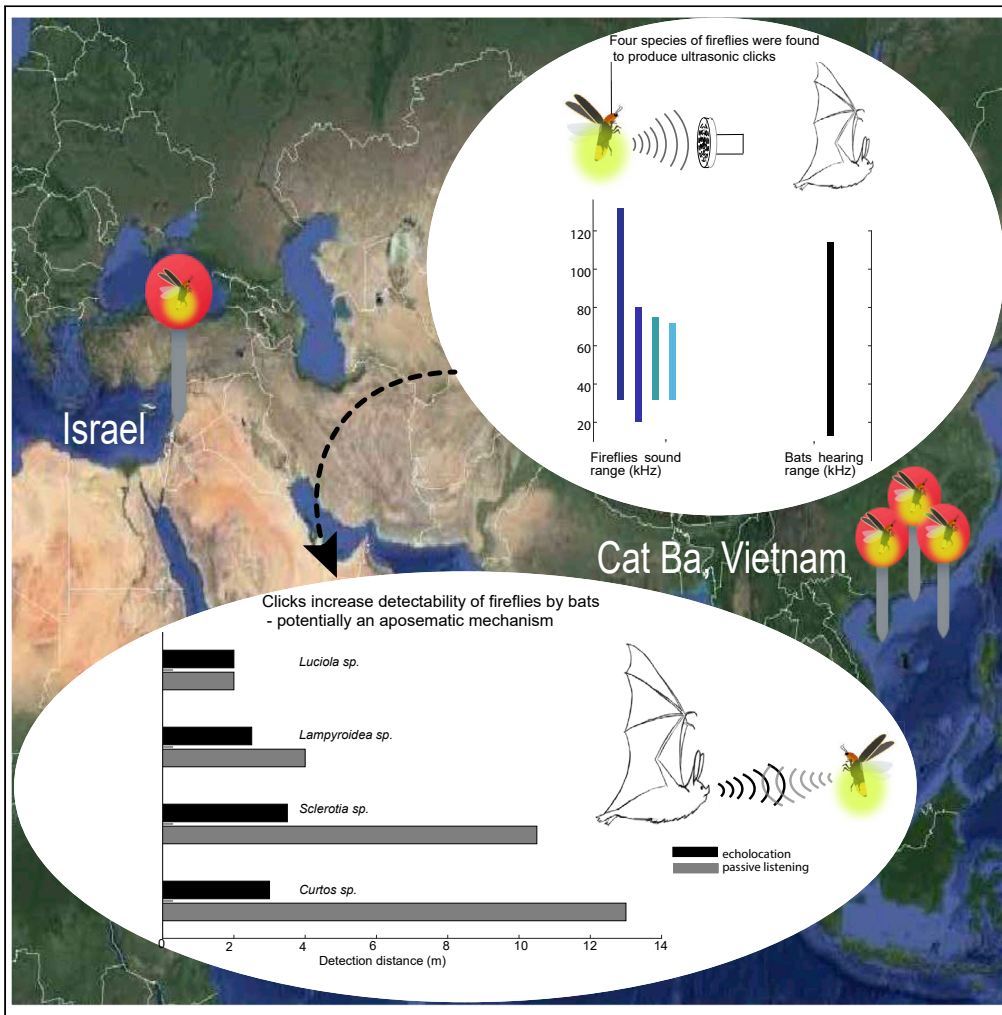


Article

Fireflies produce ultrasonic clicks during flight as a potential aposematic anti-bat signal



Ksenia Krivoruchko, Aya Goldshtein, Arjan Boonman, Ofri Eitan, Jonathan Ben-Simon, Vu Dinh Thong, Yossi Yovel

yossi.yovel@gmail.com

HIGHLIGHTS

Fireflies of four genera were found to produce ultrasonic clicks during flight

The clicks were synchronized with the wingbeats

Clicks increase detectability of fireflies by bats

The signals can potentially serve as a part of multimodal aposematic display

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Article

Fireflies produce ultrasonic clicks during flight as a potential aposematic anti-bat signal

Ksenia Krivoruchko,¹ Aya Goldshtein,^{2,6} Arjan Boonman,^{2,6} Ofri Eitan,² Jonathan Ben-Simon,² Vu Dinh Thong,^{4,5} and Yossi Yovel^{2,3,7,*}

SUMMARY

Fireflies are known for emitting light signals for intraspecific communication. However, in doing so, they reveal themselves to many potential nocturnal predators from a large distance. Therefore, many fireflies evolved unpalatable compounds and probably use their light signals as anti-predator aposematic signals. Fireflies are occasionally attacked by predators despite their warning flashes. Bats are among the most substantial potential firefly predators. Using their echolocation, bats might detect a firefly from a short distance and attack it in between two flashes. We thus aimed to examine whether fireflies use additional measures of warning, specifically focusing on sound signals. We recorded four species from different genera of fireflies in Vietnam and Israel and found that all of them generated ultrasonic clicks centered around bats' hearing range. Clicks were synchronized with the wingbeat and are probably produced by the wings. We hypothesize that ultrasonic clicks can serve as part of a multimodal aposematic display.

INTRODUCTION

Sexual signaling often increases predation risk. Although salient communication signals promote higher mating success, they might also assist predators or parasites who learn to eavesdrop on these signals to locate their prey or host (Zuk and Kolluru, 1998). The evolution of such signals is, thus, a trade-off between these two pressures. In many cases, predators evolve highly specialized sensory systems to enhance the detectability of prey communication signals (Arthur and Hoy, 2006) imposing a serious cost on their broadcast (Halfwerk et al., 2014). When the potential prey is unpalatable, however, it might evolve intra-species communication signals irrespective of the predator's sensing abilities, and under certain circumstances, it might even adapt its signals to advertise its unpalatability to potential predators (Rojas et al., 2018)

This is probably the case with fireflies, which are unpalatable or even noxious to predators (de Cock and Matthysen, 2001, 2003; Eisner et al., 1978; Fu et al., 2006, 2007; Knight et al., 1999; Long et al., 2012; Underwood et al., 1997; Vendl et al., 2016), and use bioluminescent light signals for intersexual communication (Demary et al., 2006; Forrest and Eubanks, 1995). When emitting light signals at night, fireflies expose themselves to a high risk of predation by various nocturnal predators including some bats, which, in contrast to the common belief, often rely on vision in many of their activities (Boonman et al., 2013; Danilovich et al., 2015). Previous studies suggested that firefly bioluminescence serves as an anti-bat aposematic advertisement in addition to its role in attracting mates (Goldman and Henson, 1977; Moosman et al., 2009), and a recent study showed that bats can learn to associate firefly bioluminescence with their noxiousness (Leavell et al., 2018). Some studies even suggest that bioluminescence evolved as an anti-predatory signal mechanism first (Branham and Wenzel, 2003; Martin et al., 2017).

However, light flashing might not be enough to prevent a bat from an attack. Some bats have poor vision (Bell and Fenton, 1986; Boonman et al., 2013; Suthers et al., 1969), and from some angles, the flash of the firefly might be missed. Moreover, fireflies' flight speed is low, and their flashing rate can sometimes be as slow as once every 7 s (Charles and Snyder, 1920). When also considering the short detection range enabled by echolocation, and the very rapid nature of bat attacks (Kalko, 1995; Schnitzler et al., 1987, Schnitzler et al., 1988; Vanderelst and Peremans, 2018), a bat might occasionally intercept a firefly with a blink of an eye (in between two flashes). Indeed, because of such identification mistakes, defense displays in nature evolve multimodal patterns, increasing their reception by a potential predator. For example, many diurnal insects combine distinct coloration with various sounds to deter predators (Rowe and Halpin, 2013). We

¹Department of Neuroscience, Rappaport Research Institute and Faculty of Medicine, Technion, Haifa, Israel

²School of Zoology, Faculty of Life sciences, Tel Aviv University, Tel Aviv, Israel

³Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel

⁴Institute of Ecology and Biological Resources, VAST, Cầu Giấy, Hà Nội, Vietnam

⁵Graduate University of Science and Technology, VAST, Cầu Giấy, Hà Nội, Vietnam

⁶These authors contributed equally

⁷Lead contact

*Correspondence: yossi.yovel@gmail.com

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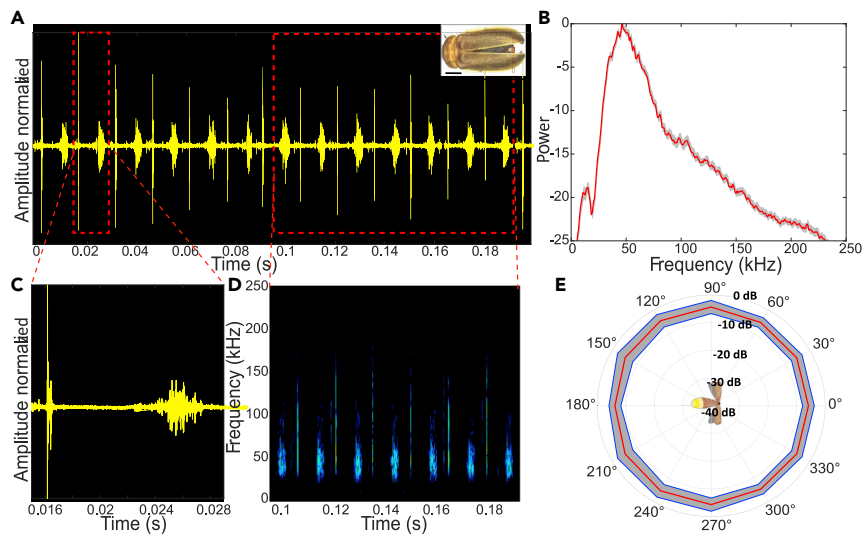


Figure 1. Acoustic characteristics of *Sclerotia*

All panels show data for the first most intense click.

(A) The waveform of the clicks. Right upper corner: photograph of the specimen. Scale bar, 2 mm.

(B) Average power spectrum of both (higher and lower clicks), mean (red) \pm SE (gray).

(C) Zoom-in on one pair of clicks.

(D) Spectrogram of the signal.

(E) Beam directionality analysis reveals that the beams are omnidirectional in the horizontal plain, mean (red) \pm SD (gray).

Intensity values were measured for the most intense frequency at a 17 cm distance.

thus hypothesized that fireflies might signal their noxiousness using more than only light. Specifically, we examined whether they also produce sounds that could be detected by bats. We examined both males and females of species of three genera of fireflies (*Sclerotia*, *Curtos*, and *Luciola*) common in Cát Bà, Vietnam. We recorded them in flight and tested their behavioral response to ultrasonic playbacks of bat echolocation calls. We found that all three species constantly produced click sounds during flight centered at bats' hearing range (Heffner et al, 2003, 2013; Koay et al, 1998, 2002, 2003; (Moss and Schnitzler, 1995)) in synchrony with their wingbeats. To examine the generality of the phenomenon, we also recorded a species common to Israel (genus *Lampyroidea*) and found that it too produces sound clicks in flight. We hypothesize that these sounds can be learned by bats and serve as an additional aposematic signal.

RESULTS

The reported experiment was performed on tethered fireflies. Initial recordings, though, were performed in a box with freely-flying (non-tethered) fireflies and also revealed clicking so that the recorded clicks were not an artifact of the tether. All Four species of fireflies that we examined produced ultrasonic clicks in tethered flight (see Figures 1, 2, 3 (see Figure S4 for setup), and Figure S1). The frequency range of the clicks ranged between 20 and 130 kHz (Figures 1, 2, 3A–3D, and S1) with peak frequencies between 40 and 50 kHz (see Table 1 for the exact frequencies). These peak frequencies overlap with the prime hearing sensitivity of a substantial proportion of bat species ((Jakobsen et al., 2013)). The intensity of the clicks at 1 m distance was similar in the two larger *Sclerotia* and *Curtos* species (59- and 60-dB SPL, respectively), but substantially weaker in the smaller *Luciola* and *Lampyroidea* species (25 and 37 dB SPL, respectively). All the SPL values were measured peak to peak. We were able to record from both sexes in two of the four species (females of *Lampyroidea* are not airborne) and found that males and females produced signals with roughly the same power density profile (Figures S2 and S3), suggesting that these signals are not used in courtship behavior.

The fireflies' clicking rate was very similar to the rate of their wingbeat, suggesting that clicks are produced by the wings (Figure 4A and see Video S1). In the two larger species, the rate of the clicks was twice higher than the wingbeat rate, with one of the clicks being quieter and slightly different in spectrum than the other (suggesting that they are produced by different parts of the wingbeat cycle). In the two smaller species the rate of clicks was roughly the same as the wingbeat rate.

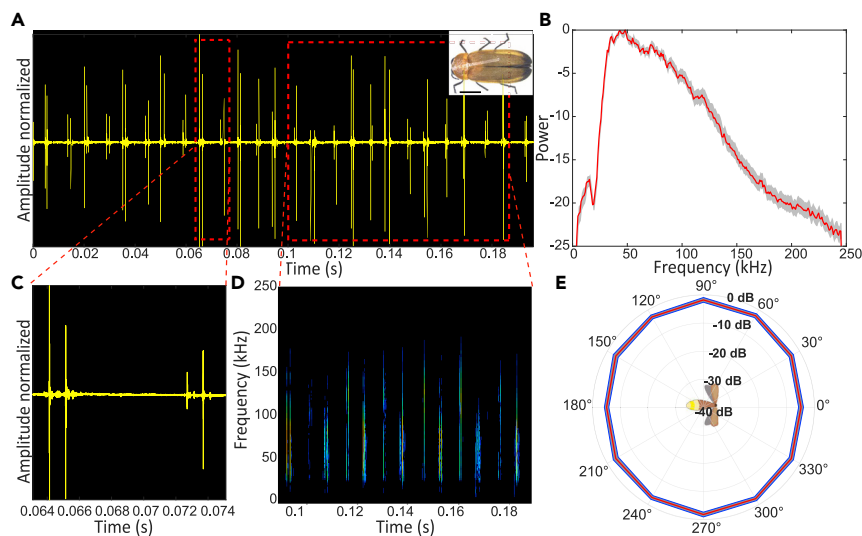


Figure 2. Acoustic characteristics of *Curtos*

All panels show data for the first most intense click.

(A) The waveform of the clicks. Right upper corner: photograph of the specimen. Scale bar, 2 mm.

(B) Average power spectrum of both (higher and lower clicks), mean (red) \pm SE (gray).

(C) Zoom-in on one pair of clicks.

(D) Spectrogram of the signal.

(E) Beam directionality analysis reveals that the beams are omnidirectional in the horizontal plain, mean (red) \pm SD (gray).

Intensity values were measured for the most intense frequency at a 17 cm distance.

An analysis of the directionality of the emission (Figures 1, 2, and 3E) showed that the click sounds spread in all directions almost equally. Therefore, bats should be able to hear the fireflies from a similar distance from all directions (at least in the horizontal plane). We estimated the maximum distances over which a bat could detect these fireflies based on their clicking to be $\sim 13.0 \pm 2.2$ m for *Curtos* and $\sim 10.5 \pm 1.4$ m for *Sclerotia*, but only $\sim 2.1 \pm 0.4$ m and 3.8 ± 0.65 m for *Luciola* and *Lampyroidea*, respectively. We also estimated the range from which a bat could detect them actively—using its echolocation—and found it to be $\sim 3.3 \pm 0.2$ m for *Sclerotia*, $\sim 3.0 \pm 0.2$ m for *Curtos*, $\sim 1.9 \pm 0.1$ m for *Luciola*, and 2.4 ± 0.2 for *Lampyroidea* (Figure 4B). Active detection ranges were estimated assuming a 110 dB dynamic range for the echolocation system (Methods). When assuming a 120 dB range, detection distances increased to 5.1 ± 0.3 m for *Sclerotia*, 4.6 ± 0.3 m for *Curtos*, 3.0 ± 0.2 m for *Luciola*, and 3.8 ± 0.25 for *Lampyroidea* (Figure 4B: mean \pm standard deviation for all of the above).

Fireflies are not known to possess tympanic or other ultrasound-sensitive ears. Moreover, a careful examination of the fireflies we studied did not reveal ear-like structure in either of the three species. To further examine their ability to hear ultrasound, we performed several tests (on the three species from Vietnam). (1) We examined whether a non-flying firefly initiates flight or flashing in response to a loud playback (ca. 70 dB SPL) of an approaching bat. (2) We tested whether a tethered flying firefly changes the characteristics of the sound it produces in the presence of the same playback. Bats typically emit calls at an intensity range of 130–110 dB SPL (Re 10 cm) (Jakobsen et al., 2013). Note that our playback is equivalent to a bat emitting at 130 dB SPL at a distance of ~ 11.5 m from the firefly. For a bat emitting at 110 dB SPL the distance would be ~ 4.5 m from the firefly. (3) We also observed the flying fireflies and looked for any behavioral indication that they can hear the sounds, such as changes in flashing rate or in posture and movement of legs or antennae (such as the ones described in [Yager and Spangler, 1997]). The fireflies of all three species showed no response in any of these tests, strongly suggesting that they cannot hear bats (and their own clicks). The intensity and the power spectrum of the clicks recorded during the bat playbacks also did not change significantly in the presence of bats' echolocation playback (Figures S2 and S3).

DISCUSSION

In this study we show that fireflies (of four different genera) produce ultrasonic clicks during flight. Behavioral experiments imply that the fireflies are not sensitive to these signals, and thus they cannot serve for

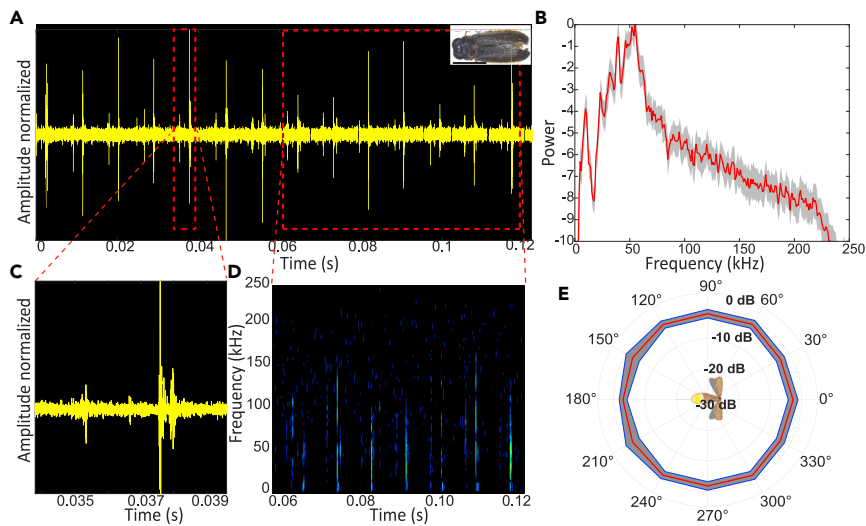


Figure 3. Acoustic characteristics of *Luciola*

All panels show data for the first most intense click.

(A) The waveform of the clicks. Right upper corner: photograph of the specimen. Scale bar, 2 mm.

(B) Average power spectrum of both (higher and lower clicks), mean (red) \pm SE (gray).

(C) Zoom-in on one pair of clicks.

(D) Spectrogram of the signal.

(E) Beam directionality analysis reveals that the beams are omnidirectional in the horizontal plain, mean (red) \pm SD (gray). Intensity values were measured for the most intense frequency at a 2 cm distance.

intra-specific communication. The main question is whether wing clicking evolved in fireflies, and more specifically, whether it evolved as an anti-bat aposematic signal? Clearly, we cannot fully answer this question without experiments that show that these clicks indeed deter bats. We cannot exclude, for example, that the clicks are an artifact of the wingbeating during flight. However, in the following paragraphs, we will discuss whether they could have evolved as aposematic signals.

Such ultrasonic clicks can be relatively easy to produce using a stridulation mechanism, for example, at the base of the wing. All firefly species that we examined clicked, and we hypothesize that most (if not all) firefly species click. Similar clicks are produced by many insects (not necessarily by the wings) for means of communication, including many species of beetles (Alexander et al., 1963; Barbero et al., 2009; Buchler et al., 1981; Conner and Corcoran, 2012; Corcoran and Hristov, 2014; Lyal and King, 1996). Some moths even evolved sound production mechanisms to disrupt bat echolocation (Corcoran et al., 2010; Corcoran and Hristov, 2014; Dunning and Roeder, 1965; MÖhl and Miller, 1976; O'Reilly et al., 2019; ter Hofstede and Ratcliffe, 2016). Although most moths do not produce wingbeat clicks in flight, some noxious moths have evolved clicking probably as an aposematic mechanism (Corcoran et al., 2010; Corcoran and Hristov, 2014; Dunning and Roeder, 1965; Hristov and Conner, 2005; MÖhl and Miller, 1976; O'Reilly et al., 2019; Rydell, 1998; ter Hofstede and Ratcliffe, 2016). Interestingly, the firefly clicks have hardly any energy below 20 kHz. Most stridulation mechanisms would generate wide band clicks with energy in audible frequencies too, so this high-frequency spectrum points toward a role of evolution. As most fireflies are noxious, if clicking evolved as an aposematic signal, it can be considered as a form of Müllerian mimicry where one species enjoys protection thanks to an encounter of the predator with another species.

If these clicks are indeed aposematic signals, why should fireflies evolve another warning signal considering their highly visible bioluminescence signals? There are several possible answers. (1) Fireflies sometimes do not emit light while flying, for example, when they fly in relatively high illumination (Firebaugh and Haynes, 2019). (2) Fireflies inter-flash interval vary between species and can sometimes be as long as 7 s (Ballantyne et al., 2013; Lloyd et al., 1989), depending on the temperature. Some studies also report lower flashing rates during flight than while perching. As explained earlier, such long intervals might result in a misidentification of the visual signal, by the hunting bats. Moreover, some bats indeed have poor vision and might miss the flashing. (3) Finally, many moths are known to signal their noxiousness using ultrasonic clicks with similar

Table 1. Summary of acoustic analyses for four species of fireflies

| Species | <i>Sclerotia</i> | <i>Curtos</i> | <i>Luciola</i> | <i>Lampyroidea</i> |
|--------------------------------|-------------------|-------------------|-------------------|--------------------|
| Firefly wing length (mm) | ~9 | ~6 | ~3 | ~5 |
| Sound intensity (dB SPL re1m) | 59 ± 2.8 (n = 6) | 60 ± 5.7 (n = 5) | 25 ± 2.7 (n = 5) | 37 ± 2.6 (n = 4) |
| Peak frequency (kHz) | 50 ± 5.3 (n = 6) | 50 ± 5.4 (n = 5) | 50 ± 6.5 (n = 5) | 50 ± 5.8 (n = 4) |
| Low frequency (kHz) | 30 ± 12.7 (n = 6) | 30 ± 9 (n = 5) | 20 ± 10 (n = 5) | 30 ± 3.9 (n = 4) |
| High frequency (kHz) | 70 ± 8.8 (n = 6) | 130 ± 5.3 (n = 5) | 80 ± 8.8 (n = 5) | 73 ± 4 (n = 4) |
| Clicking rate (Hz) | 139 ± 7.3 (n = 5) | 142 ± 14 (n = 5) | 106 ± 7.3 (n = 5) | 67 ± 7 (n = 4) |
| Wing fluttering frequency (Hz) | 69 ± 5.6 (n = 3) | 71 ± 1.1 (n = 3) | 107 ± 4.4 (n = 1) | 72 ± 4.6 (n = 3) |

Mean ± SD, n = number of sampled individuals.

High and low frequencies were defined by a drop of 6 dB relative to the frequency with maximum energy.

spectral characteristics (ter Hofstede and Ratcliffe, 2016). This includes moths that cannot hear bats, as has been recently shown (O'Reilly et al., 2019). Bats are known to avoid clicking moths (Mohl and Miller, 1976; ter Hofstede and Ratcliffe, 2016), so producing moth-like clicks (even if in a different rate) might assist fireflies to avoid predation through the act of inter-specific Mullerian mimicry.

In moths that produce aposematic clicks, it has been hypothesized that bats' detection range of these sounds should not surpass the detection range of their active echolocation (O'Reilly et al., 2019). It has been suggested that if this is not the case, bats could use the aposematic sounds emitted by these insects to localize them. In our study, the intense sounds produced by fireflies from the *Sclerotia* and *Curtos* genera enable bats to detect them from much farther than with echolocation. This was the case even though we assumed maximum insect reflectivity, which occurs when the wings are spread perpendicular to the sound wave. One explanation for these results is that the visual detection range of fireflies is mostly even larger than the acoustic detection range (depending on the visual system of the bat) so there is no point in "hiding" acoustically.

Even though all four species clicked, there were species-specific differences. The sound intensity of the two larger fireflies (*Sclerotia* and *Curtos* genera) were profoundly louder than those of the other two species. This could be an artifact of their size difference. It could also be an evolutionary outcome resulting from the difference in their active echolocation-based detection range (Figure 4B). Alternatively, the less-intense clicks could have evolved to match their different behavior. We hardly detected *Luciola* fireflies flying in the open. They were usually detected in stone crevices, in tall grass, or in tree crowns. Moreover, their flashing rate was rather high and constant, and they were usually observed in aggregations, probably lowering the significance of sound clicks as an aposematic mechanism.

The evolution of predator-prey communication is fascinating, with unpalatable prey evolving various multimodal measures of display. If ultrasonic clicks are indeed aposematic, then the combination of light flashes with ultrasonic clicks make fireflies' multimodal display of aposematism remarkably complex. Indeed, fireflies seems to avoid predation as revealed by several examinations of bat fecal pellets (the bats did consume firefly-sized beetles) (Goiti et al., 2003). Such a multimodal warning display could also assist bats' learning, helping them to distinguish truly unwanted targets from those displaying a Batesian mimicry. Additional research should focus on whether bats are deterred by these ultrasonic clicks and if so, on how they weigh different aposematic cues. All evidence suggests that fireflies cannot hear the clicks they produce. The idea of signals that cannot be sensed by their sender is intriguing. This is of course common in plants (Primack, 1982), but to our knowledge very rare in the animal kingdom.

Limitations of the study

The article has two main limitations that require further research:

- 1 .We did not perform behavioral experiments to show that firefly clicking sounds deter echolocating bats. Such experiments would be necessary to demonstrate the aposematic function of these signals.

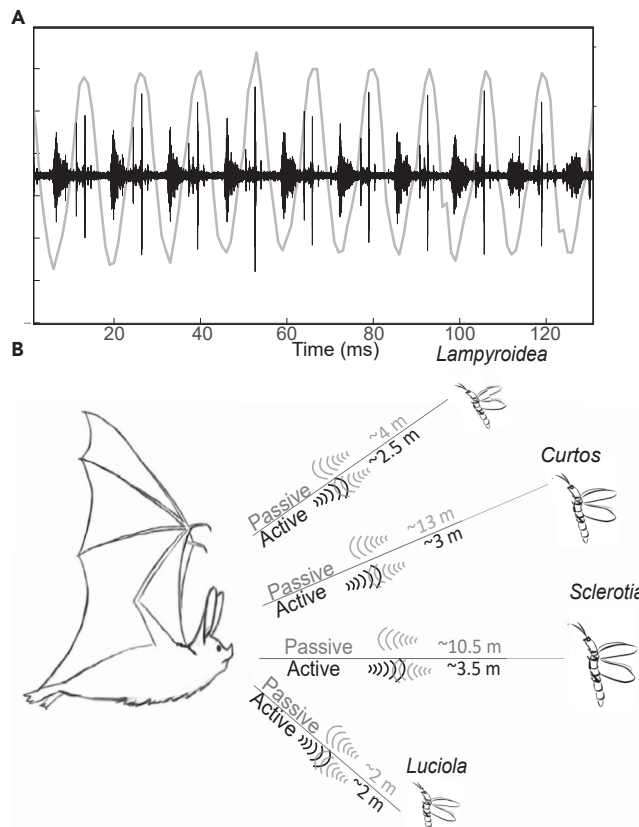


Figure 4. Click and wingbeat synchronization and click detection range

(A) Synchronization of wing movement and clicks. Wing positions are depicted by gray line: high points for wings in the upstroke positions, low points for wings in the downstroke positions. Clicks' waveforms are depicted by black line. Because our high-speed imaging and sound recordings were not synchronized, the two lines can be out of phase, but the rates are correct.

(B) The schematic describes the maximal detection range of the fireflies by a bat when relying on passive hearing of the clicks or active echolocation. This was estimated for a bat emitting a call with an intensity of 110 dB SPL (Re 1m). Estimated detection distances for 120 dB SPL are 5.1 ± 0.32 m for *Sclerotia*, 4.66 ± 0.3 m for *Curtos*, 3 ± 0.2 m for *Luciola*, and 3.8 ± 0.25 m for *Lampyroidea*.

2. We did not fully clarify the sound production mechanisms of the clicks.

Both of these are needed to better understand the evolution of the clicks.

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Yossi Yovel (yossiyovel@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original data have been deposited to Mendeley Data <http://dx.doi.org/10.17632/r3hybw89zz.1>

METHODS

All methods can be found in the accompanying [transparent methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.102194>.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.Y.; Methodology, Y.Y.; Software, Y.Y., K.K., and A.B.; Formal Analysis, K.K. and A.B.; Investigation, Y.Y., K.K., A.G., A.B., O.E., and J.B.-S.; Writing – Original Draft, Y.Y. and K.K.; Writing – Review & Editing, Y.Y. and K.K.; Project Administration, V.D.T.; Funding Acquisition, Y.Y. and V.D.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

**Fireflies produce ultrasonic clicks during flight
as a potential aposematic anti-bat signal**

Ksenia Krivoruchko, Aya Goldshtein, Arjan Boonman, Ofri Eitan, Jonathan Ben-Simon, Vu Dinh Thong, and Yossi Yovel

Transparent methods

Study site and specimens: Male and female adult specimens of three genera *Sclerotia*, *Curtos* and *Luciola* were collected in Cat Ba National Park with the permit ID 4697/UBND-NN of People's Committee of Hai Phong City, dated August 2nd, 2019. Some of the *Luciola* specimens were collected in the vicinity of the Cat Ba town. We consulted with local and international taxonomists who were only able to define the species to the genus level. The species from Israel (genus *Lampyroidea*) were collected at the north district, Mt. Carmel area. All the animals were brought into a quiet room for the recordings which were always performed on the same evening as the capture.

Audio and video recording and playback: The recording setup for fireflies collected in Vietnam (fig. S4) consisted of a 50x35x40cm³ cardboard box whose internal walls were covered with foam. Sound recordings were performed with a calibrated CM16 microphone connected to an Hm116 A/D converter (Avisoft Bioacoustics, Germany) which was positioned on one of the walls. The microphone was calibrated using a calibrated GRASS (DP40) microphone by emitting signals covering the relevant frequency range of this experiment to the two microphones when positioned as the same distance relative to the speaker. The animals were tethered above the line drawn on the bottom of the box - at 17cm (for *Sclerotia* and *Curtos*) and 2cm (for *Luciola*) from the microphone. Israeli fireflies and all moths were recorded in a large anechoic foam-covered flight room (~5.5*4.5m²). The distance from the microphone, for the Israeli species was assessed via the tracking system (composed of 16 Raptor E 1280 × 1024 pixels cameras, and four Raptor-12 4096 × 3072 pixels cameras, Motion-Analysis Corp.), with a spatial resolution of less than 1 mm (Eitan et al., 2019). Tethering was done by gluing a fishing wire to the dorsal thorax, leaving the head, wings, and elytra intact, allowing flight.

All audio recordings were performed at a sampling-rate of 500 kHz unless specifically stated otherwise. Sound analysis was performed by custom written scripts in MATLAB R2019a.

Fireflies from Vietnam were tested for a behavioral reaction to a bat playback as well. To do so, a speaker (Vifa, Avisoft) connected to an Avisoft D/A (Hm116) was positioned on the floor pointing upwards. The system was used to test hearing as follows: 1s long typical pipistrell-like attack sequences with hyperbolic Frequency-Modulated (FM) down-sweeps ranging between ~40-100 kHz were played to the firefly while it was tethered ~5-10 cm above the speaker. This sequence of FM signals represents a very broad range of echolocating bats. Playback was initiated both when fireflies were flying and when they stayed still on the tether without flashing for ~10-15s.

Video recordings of tethered flying individuals (*Sclerotia*, n = 3, *Curtos*, n = 2, *Luciola*, n = 2) were performed at 960 frames per second with an S10 phone (Samsung, Korea). Israeli fireflies (n=4) were filmed at 500 frames per second with a Sony rx100vi. Wingbeat frequency was analyzed from the slow-motion videos. The number of frames for 5 consecutive wingbeats was counted in two different time points of the videos (per individual) and the number of frames was divided by the filming rate in order to assess wingbeat frequency. The wingbeat frequency (per individual) was averaged across periods. Then the mean and SD was estimated for all individuals per species. In order to align wingbeats with clicks, the movement of the tip of the wing was tracked manually for each frame in the slow-motion video and plotted together with the audio trace (not synchronized, Fig. 4A)

Audio analysis and beam recordings: We recorded clicking intensity of the *Sclerotia* (n = 6), *Curtos* (n = 5) and *Luciola* (n = 5) specimens from 7 different angles in steps of 30° between 0° (the front of the animal) to 180° (its rear). Recordings were made twice for each angle. To this end, we rotated the tethered animal in front of the microphone while it was flying. Only the females of *Sclerotia* were recorded at 500 kHz, and therefore only those recordings were included in the analysis. For *Lampyroidea* the recordings were taken at a fixed distance, but the orientation to the microphone was not controlled. Stronger signals were taken for the further analysis. The median of the ten strongest clicks from each recording was calculated and the average value per each

direction was taken for further analysis (by taking peak intensity we essentially measure the intensity at the peak frequency). Click intensity was estimated by calibrating the Avisoft system to a calibrated 40DP 1/8-th inch instrumentation microphone. The click rate was analyzed from the Avisoft recordings (*Sclerotia*, n = 6, *Curtos*, n = 5, *Luciola*, n = 5). For each recording, the time difference between clicks was calculated for 6 consecutive clicks – 5 time intervals per recording in total. Then the mean and SD were estimated for all individuals per species.

Estimation of the active and passive detection ranges: The distance at which bats would detect the clicks produced by fireflies was calculated using the following equation:

$$cSPL - 20 \cdot \log_{10}(R/R_1) - \alpha(R-R_1) + \log 2.7 = HT \quad (1)$$

Where HT is hearing threshold of a bat (set to 20 dB SPL Re 20 μ Pascal), cSPL is the median sound pressure level of 10 clicks' (*Sclerotia*, n = 10, *Curtos*, n = 10, *Luciola*, n = 6, n – number of recordings). R is the detection distance (m), R1 is the recording distance (1m) and α is a frequency dependent atmospheric attenuation (1.6 dB/m at the most intensity emission frequency) for the average temperature and humidity levels in the area (26°C at night and 80% respectively). Note that some researchers argue that HT should be 10 or even 0 dB SPL Re 20 μ Pascal in which case the passive detection range would further increase, but we preferred an underestimate to be on the safe side.

The detection distance for using active echolocation was calculated in a similar manner, but with a two-way spherical transmission losses and atmospheric attenuation and with taking target strength into account:

$$bSPL + TS - 40 \cdot \log_{10}(R/R_1) - 2 \cdot (\alpha(R-R_1) + \log 2.7) = HT \quad (2)$$

Where bSPL is a bat sound pressure level and TS is a target strength at R1. The dynamic range, or bSPL-HT, was set to 110dB or 120 dB which reflects a typical bat dynamic range.

Target strength estimations: In order to estimate target strength, we reconstructed a 3D mesh of the *Sclerotia* specimen. To do that, we pinned the insect with its wings stretched in a natural position according to video-data of natural flight. We then used multi-use spray paint to gently spray the insect as to increase X-ray reflection strength from weakly reflecting parts such as wings. We then performed a CT scan of the insect in a micro CT scanner (with a resolution of 0.09x0.09x1mm³, Tomoscope, Synergy Twin). The generated dicom slices were merged into stl format using Amira 2.3 3D visualization software (<https://www.fei.com/software/amira-3d-for-life-sciences/>), using adaptive thresholding for each region. The stl file was first imported into Meshmixer (<http://www.meshmixer.com/>) to be cleaned internally and checked for possible mesh-errors, and then imported into Autocad 3DS Max to correct any remaining scanning artefacts by hand. The resulting object file was again imported into Meshmixer and a mesh size was selected such that the maximum length of any element was at least 6 times shorter than the smallest wavelength used in the acoustic simulations (see below). In a final step, the insect-object was imported into Meshlab (<http://www.meshlab.net/>) in which all faces were reoriented coherently, making sure that all norms were oriented into “air” and checking genus, absence of holes and two-manifoldness. We then used a finite element model (BEMFA see (Boonman et al., 2019)) to reconstruct the insect’s echoes when the wings are spread perpendicular to the sound wave (i.e., maximum reflectance). Echo intensity was estimated in BEMFA from a distance of 0.2 m at 40 kHz, 60 kHz and 80 kHz and was corrected afterwards for a reference distance of 1 m using the following equation:

$$\text{Log}_2(R/R_1) * C \quad (3)$$

Where R is the measuring distance, R1 is a reference distance and C is a spherical transmission loss constant per each doubling of the distance (6.02 dB). Target strength for the remaining species was inferred by correcting for the species relevant wing surface area. The resulting target strengths

were: -40.73 dB, -38.83 dB and -41.82 dB for *Sclerotia*, -45.53 dB, -43.63 dB and -46.63 dB for *Curtos* and -55.49, -53.6 and -56.59 for *Luciola*.

Statistics: One-way Anova was performed in MATLAB for comparing the maximal sound intensity in 7 measured directions and for comparing clicks recorded with and without the bat call playback (*Sclerotia*, n = 6, bat playback n = 6, *Curtos*, n = 5, bat playback n = 4, *Luciola*, n = 5, bat playback n = 4).

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Supplemental information

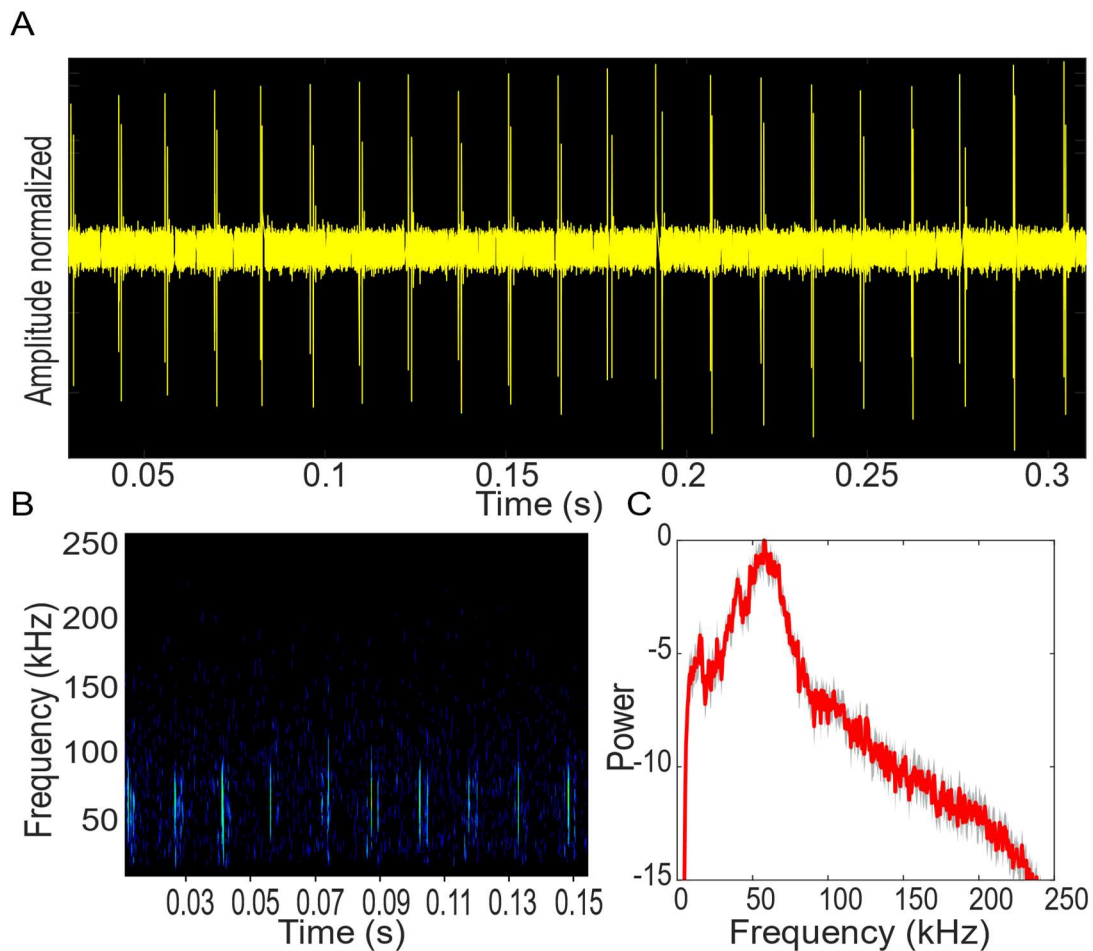


Figure S1: *Lampyroidea* recording, Related to Table 1. (A) The waveform of the clicks. (B) Spectrogram of the signal. (C) Click power spectrum – mean (red) \pm SE (grey).

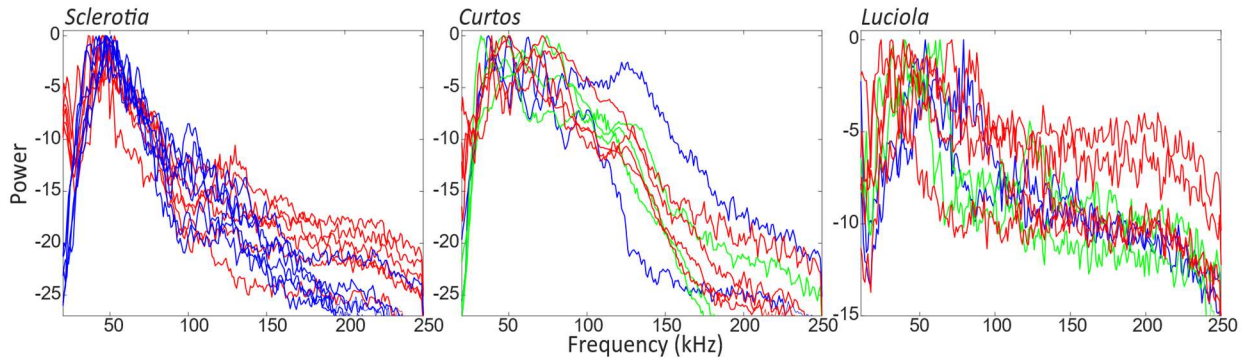


Figure S2: Clicks' power spectrum, Related to Figures 1-3. Blue lines – female individuals (*Sclerotia*, n = 6, *Curtos*, n = 2, *Luciola*, n = 2 individuals). Green lines – male individuals (*Curtos*, n = 3, *Luciola*, n = 2 individuals). Red lines – clicks of males and females together recorded during bat call playback (*Sclerotia*, n = 6, *Curtos*, n = 4, *Luciola*, n = 4 individuals).

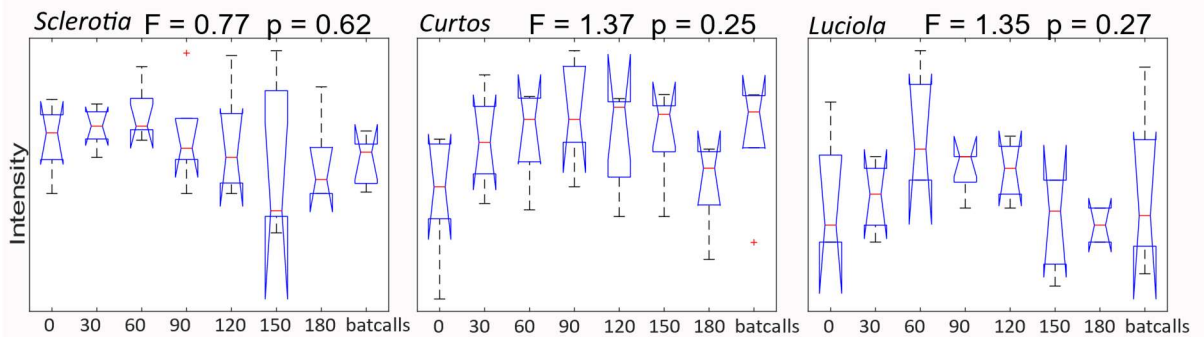


Figure S3: One-way ANOVA analysis, Related to Figures 1-3. Performed in MATLAB for maximal sound intensity in 7 measured directions and for clicks recorded during bat call playback. F-value and p-value are mentioned in the title. None of the measures has a significant difference from the rest.

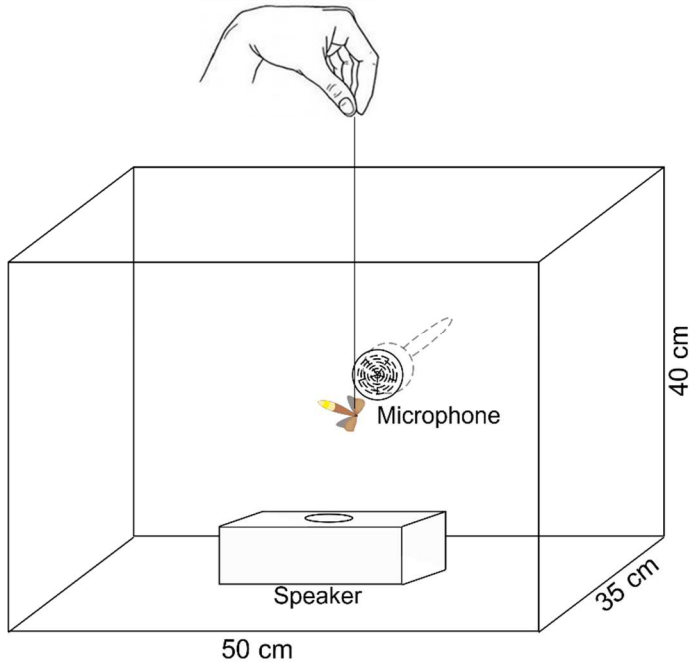


Figure S4: Setup for acoustic recordings, Related to Figures 1-3.