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Pyrazine emission by a tropical firefly: An example of chemical aposematism?

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ABSTRACT

Although famous for photic courtship displays, fireflies (Coleoptera: Lampyridae) are also notable for emitting strong odors when molested. The identity of volatile emissions and their possible role, along with photic signals, as aposematic warnings of unpalatability have been little explored, especially in tropical species. Pursuant to the observation that the widespread Neotropical fireflies, *Photuris trivittata* and *Bicellonycha amoena*, emit pungent odors, glows, and flashes when handled, we investigated their cuticular and headspace chemistry. Gas chromatography–mass spectrometry analyses revealed that both fireflies have species-specific cuticular hydrocarbon profiles. *Photuris trivittata* headspace was dominated by 2-methoxy-3-(1-methylpropyl) pyrazine (hereafter, pyrazine), on the order of 1.59 ng/individual and a suite of sesquiterpenes, while *B. amoena* emitted 3-methoxy-2-butenoic acid methyl ester and a few ketones. This is the first report of such compounds in fireflies. We investigated the role of pyrazine in *P. trivittata's* interactions with potential predators: sympatric ants, toads, and bats. Solvent-washed *P. trivittata* painted with pyrazine incurred lower ant predation than did their solvent-washed counterparts. Pyrazine significantly repelled ants at baits in concentrations as low as 9.8×10^{-4} ng/μl. The toad, *Rhinella marina,* readily accepted intact fireflies, pyrazine-coated and uncoated mealworms. Both *Myotis nigricans* and *Molossus molossus* bats rejected fireflies, but accepted both pyrazine-coated and uncoated mealworms. While pyrazine repels ants, its role as an aposematic signal warning other potential predators of firefly distastefulness requires further investigation. Our results underscore the idea that multiple enemies exert conflicting selection on firefly defenses.

Abstract in Spanish is available with online material.

Key words: *Azteca*; *Bicellonycha*; defense targeting; *Molossus*; *Myotis*; *Photuris*; *Rhinella*.

Famous for their spectacular bioluminescent courtship displays, fireflies (Coleoptera: Lampyridae) are remarkably similar to wasps and bees: both fly about with impunity, in full 'view' of numerous enemies. Often in dense aggregations, male fireflies slowly patrol for mates, while broadcasting their presence with flashes of bright light. How can fireflies display so conspicuously when surrounded by hungry birds, lizards, bats, toads, and numerous invertebrate enemies? Although larval fireflies are well known for their aposematic glow displays and their unpalatability to a variety of predators, such as mice, birds, amphibians, and fish (Lloyd 1973, Underwood *et al.* 1997, De Cock & Matthysen 2001, Fu *et al.* 2007, Vencl *et al.* 2012), our understanding of adult firefly chemical defenses remains incomplete, especially for the vast majority of species that reside in the tropics. Many well-studied North American species of the genus *Photinus* are chemically protected by steroidal pyrones known as lucibufagins (hereafter LBGs). Structurally and functionally related to other ecologically important cardiotonic and emetic toxins, such as toad bufodienolides and plant cardenolides (Eisner *et al.* 1978, Dobler *et al.* 2012, Zhen *et al.* 2012). LBGs are capable of eliciting strong rejection in vertebrate predators (Meinwald *et al.* 1979). Although most of our knowledge about firefly defenses comes from a few temperate species, virtually nothing is known about the chemical defenses of their tropical counterparts. Although fireflies reach their highest diversity in the Neotropics, the likely origin of the clade (Crowson 1981, Grimaldi & Engel 2005), evidence supporting the role of glows, flashes, noxious chemicals, or other traits that might serve as warnings of unpalatability, or that enhance resistance against an ecologically relevant, multi-species enemy milieu, is unavailable for any Neotropical firefly species.

We observed that when disturbed or attacked by predators, many firefly species common to central Panamá typically produce glandular secretions or reflexively discharge hemolymph at the same time they emit strong odors. Even in daylight, these noticeable volatile emissions are accompanied by glows and flashes. In addition, many species have a bitter taste, which is indicative of alkaloidal compounds. Thus, we hypothesized that tropical fireflies are likely defended by a diversity of noxious volatile and non-volatile chemicals, in addition to those already identified,

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such as the LBGs. Moreover, we predicted that these chemical emissions not only make fireflies distasteful to their potential nocturnal enemies, such as ants, toads, and bats (*cf.* Lloyd 1973, Eisner *et al.* 1978, Lewis *et al.* 2012), but may also serve as warnings of such distastefulness or repugnance, consistent with an aposematic defense strategy.

We compared the mixtures of cuticular and volatile organic compounds emitted by the widespread and sympatric Neotropical fireflies, *Photuris trivittata* and *Bicellonycha amoena*. We then assessed the acceptability of these fireflies to common nocturnal predators (ants, toads, and bats), and then tested the possible defensive effects of one major volatile, 2-methoxy-3-(1-methylpropyl) pyrazine (hereafter, pyrazine) emitted by *P. trivittata*. Pyrazines are ubiquitous odorants released by numerous insect species, which often function as alarm or as warning signals that enhance the protective effects of warning coloration or noxious chemical defenses (Moore *et al.* 1990, Rowe & Guilford 1999, Vander Meer *et al.* 2010). Together, the multi-tiered approach of our study addresses the question of whether several enemies, with different modes of attack, can impose concerted or perhaps conflicting selection on the evolution of prey defenses. Distinguishing between these selective outcomes could help explain the role of chemical diversity in the evolution of firefly defensive arsenals during the radiation of the clade.

METHODS

Study site.—Between 2011 and 2014, fireflies, bats, and toads were collected in the vicinity of Gamboa (9°07′6″ N, 79°42′5″ W: 48 m asl), a small town surrounded by late secondary rain forest of the Soberanía National Park, Republic of Panamá. Bioassays were conducted in nearby forest (ants), ambient laboratories (toads), and in flight cages (bats) of the Smithsonian Tropical Research Institute.

Photuris trivittatta (Lloyd & Ballantyne 2003) and *B. amoena* (Gorham 1880) are widespread firefly species commonly found in Neotropical lowland rain forests. *Bicellonycha amoena* is found from Mexico to Panamá (Fig. 1A), while *P. trivittata's* range extends from Mexico to Colombia (Fig. 1B; Lloyd & Ballantyne 2003). Both species frequent open areas within or adjacent to secondary forest at elevations below 500 m. Female *Photuris* fireflies, known as '*femme fatales*', are specialist predators on males of other firefly species: *femme fatales* use aggressive mimicry of courtship flash patterns to attract and capture male prey in order to sequester defensive toxins they cannot produce themselves (Fig. 1B; Eisner *et al.* 1997). Both species are active from just after dusk into early evening (1900–2200 h). Fireflies used in chemical analyses and in feeding tests were collected the evening before experiments and maintained at ambient temperature in plastic cups with moist grass.

Elucidation of firefly cuticular and volatile organic compounds.—We extracted cuticular (non-volatile, cuticle-bound) mixtures by immersing freshly frozen *P. trivittata* ($N = 8$) and *B. amoena* ($N = 6$) individual firefly adult males for 15 min in 0.5 or 0.1 ml of methylene chloride, respectively. We then evaporated the solvent with flowing nitrogen gas, and re-dissolved extracts in

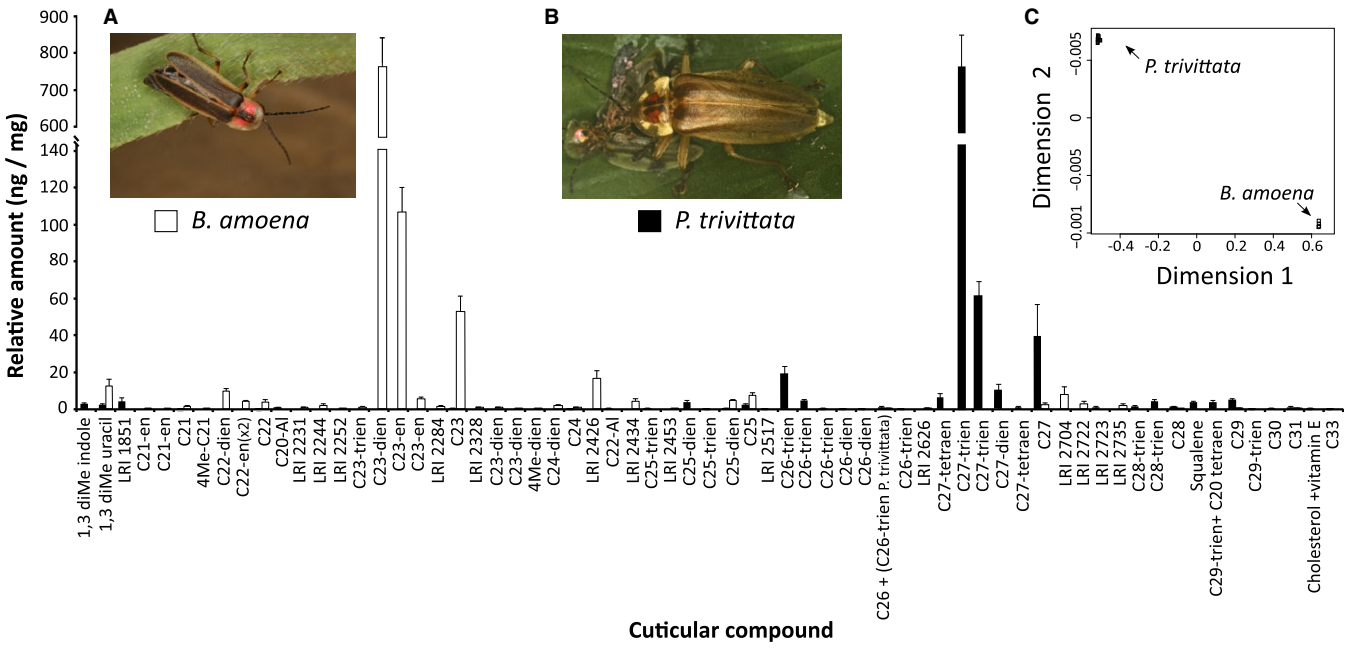

FIGURE 1. Composition of cuticular compounds extracted from (A) *Bicellonycha amoena* (white bars) and (B) *Photuris trivittata* (black bars), which has captured and is consuming a male *Aspisoma* firefly. A linear retention index value (LRI) is given for unidentified compounds. Insert C shows the similarity in composition of the cuticular mixture of both species (nMDS stress = 0.007%).

50 μl of methylene chloride and 180 ng of tridecane, the latter used as an internal standard.

We sampled all volatile emissions surrounding the firefly body, hereafter referred to as 'headspace' volatiles, of live *P. trivittata* and *B. amoena* adults, using solid phase micro-extraction (SPME), with a 65 μm polydimethylsiloxane/divinylbenzene fiber (Supelco; Sigma-Aldrich Corporation, St. Louis, MO, U.S.A.). We conditioned fibers at 250°C for 30 min and then exposed them to the fireflies for 2 h at room temperature (*ca* 24°C). We sampled the headspace of ten groups of four *P. trivittata* and three groups of seven *B. amoena* inside a 4 ml glass vial fitted with a polyprolylene cap with a PTFE septum. We also added a plug of glass wool to vials (76 ± 6 mg; mean ± SE) to prevent the fireflies from touching the SPME fiber. Samples from vials containing glass wool alone were used as a control to eliminate background odors from the firefly samples.

We analyzed the headspace samples and cuticular extracts by gas chromatography–mass spectrometry (hereafter, GC-MS) using an Agilent gas chromatograph 6890N, equipped with either a DB-5, or an HP5-ms fused silica capillary column (30 m × 0.25 mm, 0.25 μm), connected to a 5973 Network mass selective detector. We injected in splitless mode, at 250°C, using helium as the carrier gas (constant flow of 1 ml/min). The temperature program started at 50°C, held for 1 min, then rose to 200°C at a heating rate of 6°C/min, and then finally to 300°C, with a heating rate of 10°C/min. This maximum temperature was held for 5 min. We report compounds that appeared in at least two of the firefly samples for either volatile or cuticular mixtures.

We did a tentative identification of compounds by comparison between observed peak mass spectra with those from data bases and published literature (Joulain & König 1998). We then calculated linear retention indices (LRI) for each compound and compared the obtained values with those reported elsewhere (Adams 2001, NIST 2015). The identification of many compounds using mass spectra alone is limited, particularly for sesquiterpenes, which were common in our samples (Merfort 2002). Therefore, putative names for compounds were given when we obtained both a match higher than 90 percent with the Wiley Registry of Mass Spectral data (7th edition), and also a close match with published retention indices. For the latter, values of less than ±10 RI units were considered an accepted match (D'Acampora Zellner *et al.* 2008). We used LRI values reported by Adams (2001). When these were not available, we used the most frequent index for the compound given in the NIST Chemistry WebBook (2015).

We confirmed the identity of 2-methoxy-3-(1-methylpropyl) pyrazine with a commercial reference standard (≥98%, Sigma-Aldrich Corporation), and quantified the amount released by *P. trivittata* using external standardization. We prepared solutions of 9, 0.9, and 0.09 ng/μl of the standard compound dissolved in ethanol. Then, we created a calibration curve with the peak area detected in four samples of the headspace of 10 μl of each solution added to a paper filter disk. We sampled the headspace of the infused paper filter and a plug of glass wool for 2 h in a 4 ml vial with SPME and GC-MS using identical conditions as those used for sampling live insects.

We compared the composition of methylene chloride cuticular extracts of both firefly species using non-metric multidimensional scaling (nMDS). For these, we calculated the rank order of pairwise, Bray–Curtis distances among samples based on the square root-transformed relative abundance of each component of the chemical mixtures. The minimal stress achieved measured how well distances between samples were represented by the ordination, with a value below 10% commonly considered a good fit (Zuur *et al.* 2007). We further performed a multivariate, nonparametric analysis of dissimilarities, using Adonis, to detect statistical differences in cuticular composition among species. For these tests we used the R package Vegan (Oksanen *et al.* 2013).

Ant bioassays.—We used a common and aggressively recruiting generalist ant predator, *Azteca lacrymosa* Forel (Hymenoptera: Formicidae: Dolichoderinae) in field bioassays (*cf.* Vencl *et al.* 2011). A test arena was made from a platform, measuring 45 cm × 60 cm, attached 60 cm above the ground to the bole of a tree with an *A. lacrymosa* nest. Vines and fallen branches connected the platform to the nest, such that the ants formed active, clearly defined trails across the platform's surface.

Two types of no-choice bioassays were used to examine ant responses to fireflies and their headspace volatiles, particularly *P. trivittata*'s pyrazine. In the first type of bioassay experiment, freshly frozen (−2°C for 10 min) adult *P. trivittata* ($N = 40$) were randomly divided among the following four treatment groups: (1) unwashed (intact); (2) methanol (MeOH) washed; (3) MeOH-washed + 0.9 ng/μl pyrazine, and; (4) MeOH-washed + distilled water control. For those fireflies not in the intact group, adults were washed in 5 ml MeOH for 2 min and allowed to air-dry for 10 min. A solution of the commercial standard of 2-methoxy-3-(1-methylpropyl) pyrazine (Sigma-Aldrich) was applied to the fresh-frozen adults with a pipetter 3 min prior to the bioassay experiment. We dissolved pyrazine in distilled water because ants rejected mineral oil, which was used in tests with other predators (see below). For each trial, we used soft forceps to place a single adult firefly 1 cm from an active foraging trail on the platform. A trial began after the first ant antennated the firefly. A firefly was deemed captured when the ants carried it ≥1 cm toward the nest. Using a digital stopwatch, we quantified capture time as the interval from the first ant contact (trial onset) to the movement of the treatment item (trial end). If the firefly was not captured, the trial ended in 5 min. Individual trials were conducted along different trails on the platform and were separated from one another by at least 5 min. Ant bioassays of *P. trivittata* were conducted during the early wet season from June to July 2012, between 0800 and 1130 h.

We examined capture times of *P. trivittata* fireflies using failure-time statistics as implemented in PROC LIFETEST (SAS 2004). In contrast to classical methods, such as ANOVA that compare either the total number of captures at the end of the experimental time interval, or the average capture time among treatment groups, failure-time methods compare the distributions

of capture times throughout the entire bioassay period. The time to the occurrence of an event (*e.g.*, capture of a firefly by an ant) does not typically meet the distributional assumptions required by traditional parametric approaches. In addition, many of the trials ended before a capture event was recorded (*i.e.*, right-censored data) and the ultimate fate of the firefly beyond the bioassay interval was unknown. Capture functions were compared using Wilcoxon's signed ranks test followed by pairwise multiple comparisons to determine specific differences between treatment groups (Kalbfleisch & Prentice 1980). Significance levels were corrected with the sequential Bonferroni technique (Dunn–Sidák method; Sokal & Rohlf 2012). This method is less conservative than the standard Bonferroni technique but ensures that an appropriate experiment-wise error rate ($\alpha = 0.05$) is maintained.

We also examined the acceptability of adult *B. amoena* fireflies to ants. Following the bioassay protocol above, we exposed MeOH-washed ($N = 20$) and intact ($N = 20$) *B. amoena* to ant attack in June 2014. Capture frequencies of intact and solvent-washed *B. amoena* were analyzed using a 2×2 contingency table and the χ^2-test. The Yeats continuity correction was applied when cell counts were less than five to obtain a more conservative, adjusted test.

In the second type of bioassay, we tested the effect of pyrazine alone on the ant feeding behavior. We prepared liquid bait consisting of a 1 M sucrose solution using distilled water. From a stock pyrazine solution derived from the commercial standard (see above), six test solutions ranging from 9.8 to 9.8×10^{-7} ng/μl were prepared by dilution with distilled water. A bioassay trial consisted of pipetting 10 ml of the stock sucrose solution into a 4 cm Petri dish on the test platform. After 20 min, we photographed the dish to quantify the number of ants feeding at the bait. A 200 μl aliquot of a given pyrazine test solution was then pipetted into the center of the dish. A second photo was taken after 10 sec to record the number of ants at the treated bait. Between 22 and 38 replicate before–after sucrose bait trials were deployed at each pyrazine concentration. An injection of 200 μl of distilled water into a sucrose bait dish served as a control. Successive treatment or control dishes were placed on the platform at different locations more than 20 cm apart and at a minimum of 5-min intervals to prevent recruitment. Recruitment occurs when an individual ant signals to others the presence of food or enemies to the effect that they join or follow the signaling ant. Such potential cross-talk between treatments would have confounded the independence of the trials. We compared the number of ants before and after the introduction of a test solution or the addition of water to the sucrose bait in the control using a paired *t*-test.

Bat bioasssays.—Experiments were conducted in December 2012. We used two vespertilionid insectivorous bat species, *Myotis nigricans* ($N = 10$) and *Molossus molossus* ($N = 7$) in a bioassay to detect the acceptability of intact fireflies and pyrazine to bats. Bats were captured in mist nets as they exited their roosts between 1815 and 2030 h. They were kept separately in cloth bags and were allowed to acclimate to captivity for at least 15 min before being hand-fed between three and seven mealworms (*Tenebrio molitor*). Bats that did not readily accept mealworms were excluded from the experiment. Trials began at least an hour after the initial screening to ensure consistent motivation. All experiments were video-recorded with a Sony® Handycam™ DCR-SR45 digital camcorder in Nightshot mode illuminated with a 25W red light bulb and two Wisecomm® IR045 Infrared LED lights.

Experiments consisted of five presentation trials in which individual handheld bats were offered with forceps either untreated mealworms, treated mealworms, or live fireflies. Behavioral responses were recorded. The five presentation trials were sequential: (1) an untreated mealworm; (2) a mealworm coated with mineral oil; (3) a mealworm coated with 0.9 ng/μl pyrazine in mineral oil; (4) a live *P. trivittata* or *B. amoena* firefly; and (5) a second untreated mealworm to ensure that the bat was still motivated to feed. Trial order was kept constant with the exception of trials (2) and (3), which were randomized between bats. Mineral oil was used as a retention medium to prolong pyrazine volatilization (Vander Meer *et al.* 2010). If a bat bit the offered item within 30 sec, it was allowed to continue eating and the amount of prey eaten was recorded. If the bat failed to contact the test item within 5 min, the trial was concluded and scored as 300 sec, mirroring the trial time period used in the ant bioassays, and the next test item was presented. If a bat failed to eat the final untreated mealworm, indicating lack of motivation to feed, the experiment was not counted. Due to limitations in the availability of wild-caught fireflies, we were not able to offer both species of firefly to each individual predator. Using Video Edit Master software (Osman 2011), trials were analyzed for latency to bite, chewing (handling) time, percentage of the trial duration spent eating, percentage of each prey item eaten, and number of head shakes. All bats were released on the same night at their location of capture.

Cane toad bioassays.—Adult male toads (*Rhinella marina*, formerly *Bufo marinus*) were collected between 1900 and 2300 h during July 2013 ($N = 16$) and June 2014 ($N = 22$). Adult males are readily distinguished by a length >9 cm, solid coloration, and by prominent nuptial thumb pads (Zug & Zug 1979). No adults were actively mating or calling when captured. After capture, toads were housed in individual plastic containers lined with wet paper towel. Following Candler and Bernal (2015), toads were given two mealworms in their home containers and individuals were tested on the fourth night after eating both mealworms, a pattern consistent with their natural feeding behavior (Zug & Zug 1979).

To examine cane toad responses to fireflies, individual toads were placed in a plastic experimental arena (33 × 40.5 × 16.5 cm) covered with mosquito netting to prevent escape but allowing video recording using a Sony HDR-UX20 camcorder set at its night function (IR light). Following the methods described for bats, an experiment consisted of the sequential presentation of untreated mealworms, treated mealworms and fireflies dropped into the arena containing a single toad. Each toad was presented a sequence of five test items as per the bat protocol above. If the

last mealworm was not consumed, the experiment was not counted. Videos were analyzed for latency to bite, total trial time, and behavior indicative of an adverse response (mouth clawing, regurgitation, rapid blinking). Of the 38 adult toads collected, 25 successfully completed all trials (11 in 2013 and 14 in 2014).

The response of juvenile cane toads ($N = 15$) to fireflies was also investigated to explore the potential role of age in aversion to fireflies. Juveniles were captured and housed in the same conditions as the adults. Toads <9 cm long, multi-colored, and lacking nuptial pads were considered sexually inactive juveniles (Zug & Zug 1979). Juveniles were only offered the smaller *B. amonea* fireflies because *P. trivittata* fireflies were too large to elicit toad attacks. As juveniles refused to feed in the experimental arena, fireflies were presented in their individual home tanks where the toads could be exposed to the prey for a prolonged time in a familiar environment. Home tanks were the same size as the experimental arena but were covered with white plastic instead of mosquito netting. Hence, their behavior could not be recorded. Fireflies were introduced into the home tanks in the evening and were checked the following day for firefly consumption. As a control for feeding motivation, each toad was given a mealworm the nights before and after a firefly presentation and its presence or absence was scored the next morning. Therefore, only intact mealworms and fireflies were presented to juvenile toads. Mealworms coated with oil or pyrazine were not included in these trials given that the prey was present in the container with the toad overnight and that the worms lost their coatings before the end of the trial. Therefore, consumption of those mealworms could be misleading. Following the experiments, toads were marked by toe-clipping to avoid recapture and then released at the collection site according to recognized guidelines (Beaupre *et al.* 2004).

We performed independent repeated measures as general linear models to examine the response variables across trials in cane toads and insectivorous bats as implemented in SYSTAT (v. 13.1; Wilkinson 2010). Because we used two species of bats, species was included as an additional independent variable for the bat analyses. Variables analyzed for bats were: latency to feed; total chewing time; percentage of time spent eating; percentage of prey item eaten; and number of head shakes. χ^2-test analysis was used to determine differences in proportions of fireflies eaten and all other prey types eaten. Latency to bite was the only variable analyzed for adult toads because no aversive behaviors were observed during experimental trials. A Fisher's exact test was used to compare the proportions of juvenile cane toads that ate fireflies and mealworms.

RESULTS

Firefly cuticular and volatile organic compounds.—Methylene chloride extracts of *P. trivittata* and of *B. amoena* cuticle were dominated by saturated and unsaturated straight chain hydrocarbons, whose lengths ranged between 21 and 33 carbons (Fig. 1; Table S1). Cuticular mixtures also included a few branched alkanes with nitrogen-containing rings, aldehydes, and several unidentified compounds. Although *P. trivittata* and *B. amoena* shared several compounds, particularly shorter alkanes, their cuticular mixtures were significantly different (Adonis: R2 = 0.93; $F_{1,12} = 174.5$, $P < 0.001$) and separated extremely well in the nMDS tests with a 0.007 percent stress value (Fig. 1C). The *P. trivittata* compound profile was dominated by heptacosane and three C27 alkenes, altogether comprising about 90 percent of the total mixture. In contrast, the cuticular mixture of *B. amoena* was dominated by tricosane and two C23 alkenes.

Solid phase micro-extraction samples of the headspace of *P. trivittata* showed 35 volatile compounds, 28 of which were tentatively identified, which consisted primarily of sesquiterpenes and sesquiterpene alcohols (68%), followed by methoxy-pyrazines and a few alcohols, ketones, and alkanes (Table 1; Table S2). Two of the three methoxy-pyrazines present, 2-methoxy-3-(1-methylethyl) and 2-methoxy-3-(1-methypropyl) pyrazine were consistently detected in all samples. We estimated that individual *P. trivittata* released about 1.59 ± 0.1 ng (mean ± SE) of the latter compound in our headspace vials and tested its effect on the feeding behavior of potential firefly predators with bioassays (Fig. S1). In contrast, SPME samples of *B. amoena's* headspace contained far fewer volatile compounds, which included 3-methyl-2-butenoic acid, methyl ester, three ketones, an alcohol, and a single sesquiterpene (Table 2). Only the alcohol, 1-octanol, was also a component of *P. trivittata's* volatile mixtures. Headspace samples of *P. trivittata* contained 1,3-dimethyl uracil, a compound that was also found in cuticular extracts of both fireflies species (see Fig. 1; Table 1).

Ant bioassays.—*Azteca* ants took significantly longer to capture both intact and pyrazine-augmented *P. trivittata* fireflies than the solvent-washed fireflies. The pyrazine treatments also had longer survival times (time to capture) and higher survival frequencies (percentages) than the solvent-washed treatment during the 5-min bioassays (Fig. 2). There was no difference in survival time between intact fireflies and solvent-washed fireflies with a topical application of 0.9 ng/μl pyrazine. Ants slowly approached pyrazine-treated fireflies before contacting them. The ants antennated the fireflies briefly, often retreating and circling several times before approaching them to make contact with their mouthparts or leave without assuming a recruitment posture (abdomen tip down and touching substrate). Ant responses to the *B. amoena* with and without its chemical compounds were consistent with those observed for intact and MeOH-washed *P. trivittata*. Although solvent-washed *B. amoena* fireflies readily fell prey to ants, significantly fewer intact fireflies were captured during the bioassay (85%; 40%, respectively; χ^2-test = 8.64, $P = 0.0033$).

The addition of standard pyrazine to the sucrose bait in concentrations ranging from 9.8 to 9.8×10^{-4} ng/μl significantly reduced ant feeding in a nearly linear dose-dependent relationship (Fig. 3). Immediately upon administration of pyrazine, ants fled but began to return to baits within 3 min, indicating that pyrazine's repellent effect was short-term. The repellent effect of pyrazine was not detectable at or below a concentration of 10^{-5} ng/μl (Fig. 3).

Bat bioassays.—Bats rejected 84 percent of the proffered fireflies (16/19; 0 sec chew time) and rejected fireflies more often than

TABLE 1. *Volatile compounds emitted by Photuris trivittata fireflies.*

Compound[a]	LRI DB-5[b]	LRI literature[b]	Occurrence (%)[c]	Area ($\times 10^5$) ($\pm$SE)[d]
1-Hexanol	867	867	80	16.671 ± 6.455
2-Heptanone, 6 methyl	955	–	90	9.512 ± 3.343
Nonane, 4,6-dimethyl	1012	–	40	1.500 ± 0.949
2 Ethyl hexanol	1024	1030	40	6.995 ± 4.441
2-Heptanone, 4,6-dimethyl	1048	*1045*	90	4.606 ± 0.859
1 Octanol	1067	1070	80	1.811 ± 0.375
Pyrazine, 2-methoxy-3-(1-methylethyl)	1091	1097	100	7.275 ± 1.813
Unidentified M^+166	1159	–	100	3.538 ± 1.205
Pyrazine, 2-methoxy-3-(1-methylpropyl)	1173	1176	100	4.435 ± 0.890
Pyrazine, 2-methoxy-3-(2-methylpropyl)	1182	1180	80	0.442 ± 0.207
δ-Elemene	1342	1339	50	0.473 ± 0.218
Cyclosativene	1374	1368	60	4.953 ± 1.829
α-Ylangene	1378	1372	30	0.184 ± 0.184
1, 3-dimethyl uracil	1392	–	50	1.892 ± 1.648
1, 5-di-epi-β-Bourbonene	1392	1390	20	0.591 ± 0.374
β-Cubebene	1396	1390	40	Eluted with β-Elemene
β-Elemene	1397	1391	40	4.047 ± 1.501
β-Caryophyllene	1427	1418	80	10.032 ± 4.674
Unidentified sesquiterpene ($C_{15}H_{24}$)	1432	–	20	0.976 ± 0.624
Unidentified sesquiterpene ($C_{15}H_{24}$)	1437	–	30	Eluted with γ-Elemene
γ-Elemene	1439	1433	40	1.491 ± 0.711
trans-α-Bergamotene	1441	1436	20	1.725 ± 1.725
α-Humulene	1462	1455	50	0.951 ± 0.513
4,5-di-epi-Aristolochene	1477	1470	40	1.416 ± 0.916
Unidentified alkane	1482	–	30	1.860 ± 1.073
Selina-4,11-diene	1483	1482	90	2.783 ± 1.801
Germacrene D	1489	1480	70	5.605 ± 2.227
Eremophila-1(10),7-diene	1495	1488	70	2.141 ± 1.650
Unidentified sesquiterpene ($C_{15}H_{24}$)	1501	–	50	Traces
α-Muurolene	1506	1499	50	1.343 ± 0.490
γ-Cardinene	1522	1513	40	1.594 ± 0.562
ω-Cadinene	1529	*1526*	80	3.352 ± 1.665
Unidentified sesquiterpene ($C_{15}H_{26}O$)	1537	–	60	8.227 ± 3.540
Benzothiazole, 2-(methylthio)	1603	–	30	1.911 ± 1.485
Unidentified sesquiterpene ($C_{15}H_{26}O$)	1621	–	20	0.577 ± 0.383

[a]Tentative molecular formula or molecular ion (M^+) of isolated compounds. The major peaks of the EI mass spectrum for these compounds are given in the Table S2. Putative compound names are given when there was both a match higher than 90% with the Wiley data base and with published retention indices.

[b]Linear retention indices reported for compounds analyzed on DB-5 or on HP-5ms (*italics*) columns (Adams 2001, NIST 2015).

[c]Percentages of samples in which each compound was detected ($N = 10$).

[d]Mean peak areas calculated per individual with samples analyzed with the DB-5 column.

any other proffered test item (χ^2-test = 51.05, $P < 0.0001$). The behavior of the bats when eating also suggested that fireflies were treated differently from other prey. There was a difference in individual latencies to bite across trials (Fig. 4A; $F_{4,56} = 5.799$, $P < 0.001$), with the bats taking longer to bite fireflies than any of the other prey types; the two species did not differ from each other in their latency across trials ($F_{1,14} = 1.030$, $P = 0.327$). Chewing time reflects acceptance of a prey type: items chewed for a short time were quickly rejected (dropped) while items chewed for a long time were usually consumed in their entirety. Chewing time differed among trials as well as between species (Fig. 4B; $F_{4,56} = 18.608$, $P < 0.0001$; $F_{1,14} = 12.721$, $P = 0.003$, respectively). *Myotis* individuals spent more time chewing than *Molossus* individuals in all trials except the second uncoated mealworm. As most individuals did not eat the proffered firefly, time spent chewing this prey was significantly the shortest, which again indicates the strong rejection of both firefly species by both species of bats.

TABLE 2. *Composition of volatile compounds detected in the headspace of Bicellonycha amoena.*

Compound[a]	LRI HP5-ms[b]	LRI literature[b]	Peak area (%)[c]	Area ($\times 10^5$) (±SE)[4d]
3-Methyl-2-butenoic acid methyl ester	802	842	81.67 ± 19.38	100
2-Heptanone	870	880	3.15 ± 2.76	66
2-Octanone	985	993	1.37 ± 1.20	66
2-Ethyl hexanol	1024	1028	11.21 ± 14.12	100
2-Nonanone	1090	1090	0.86 ± 1.49	66
Unidentified	1475	–	0.48 ± 0.47	66
Unidentified sesquiterpene ($C_{15}H_{24}$)	1532	–	0.48 ± 0.43	100
Unidentified	1677	–	0.78 ± 0.90	66

[a]The major peaks of the EI mass spectrum for these compounds are given in Table S2.

[b]Linear retention indices reported for compounds analyzed on the HP-5ms column and in the literature (NIST 2015).

[c]Percentages of samples where each compound was detected ($N = 3$).

[d]Mean peak areas.

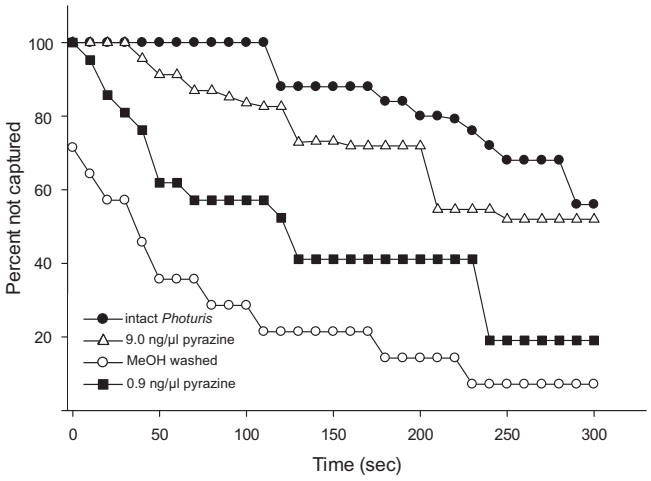

FIGURE 2. Capture curves for freshly killed, methanol leached, and 2-methoxy-3-(1-methylpropyl) pyrazine-augmented *Photuris trivittata* in the *Azteca* ant field bioassay ($N = 10$ per treatment). Error bars eliminated for clarity.

Total chewing time and percentage of time spent eating were strongly correlated ($R^2 = 0.129$; $P < 0.001$). In agreement with the results about time spent chewing the different prey items, pairwise comparisons among trials show that the percentage of trial duration spent feeding was significantly shorter with fireflies (6.76 ± 6.24% sec) than for any other treatment item: first mealworm (86.83 ± 4.88% sec, $P < 0.0001$); pyrazine-coated mealworm (84.02 ± 6.51% sec, $P = 0.0092$); oil-coated mealworm (92.79 ± 2.87% sec, $P < 0.0001$); final mealworm (70.50 ± 10.22% sec, $P = 0.024$).

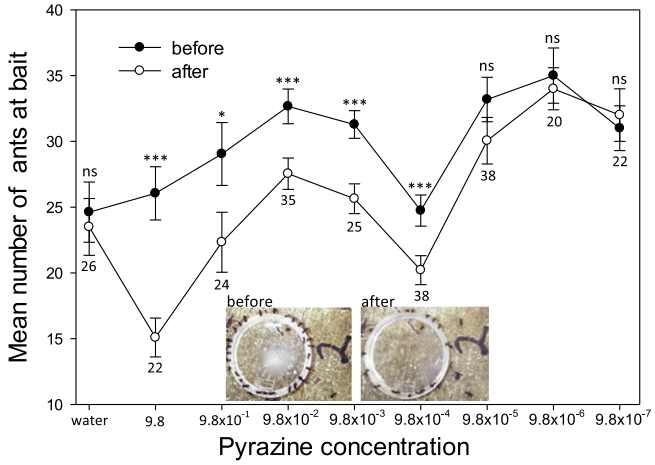

FIGURE 3. Effect of 2-methoxy-3-(1-methylpropyl) pyrazine on *Azteca* ants feeding at sugar baits. Value below each mean gives the number of before-after trials (paired *t*-test: $*P < 0.05$, $***P < 0.001$). Inset: *Azteca* ant bioassays before and after 9.8 × 10^{-3} ng/µl pyrazine was added to the sugar bait.

When proffered some items, bats shook their heads rapidly and repeatedly tried to avoid direct contact with the test item. We interpreted head shake behavior as the rejection of the presented item. The number of head shakes differed among trials ($F_{4,56} = 3.794$, $P = 0.008$) such that the highest number of shakes occurring in response to fireflies. There were no differences in the number of head shakes across trials between bat species ($F_{1,14} = 0.054$, $P = 0.819$). The high rate of firefly rejections in both bat species explains the consistently low chewing time, shorter trials, and smaller percentages of feeding time for both bat species in response to fireflies.

The amount of prey consumed by the bats differed among trials ($F_{4,56} = 41.737$, $P < 0.0001$) and between species across trials ($F_{1,14} = 13.274$, $P = 0.003$). Pairwise comparisons among trials show that less of the firefly was eaten (6.3 ± 6.3%) than any of the other prey items: first uncoated mealworm (97.8 ± 1.64%, $P < 0.0001$); pyrazine-coated mealworm (87.5 ± 7.57%, $P < 0.0001$); oil-coated mealworm (99.4 ± 0.63%, $P < 0.0001$); second uncoated mealworm (58.1 ± 11.64%, $P < 0.001$). We find parallel patterns for the amount of prey consumed and the time spent chewing, indicating correlation between these two variables.

Toad bioassays.—Overall, cane toads were equally fast at responding to and eating all prey types: mealworms with or without pyrazine, and intact fireflies. As noted above, juvenile toads did not respond to prey presented to them in the experimental arena. Individuals continually jumped at the mesh covering of the arena and attempted to climb the walls. These behaviors suggest anxiety rather than lack of hunger or foraging motivation. All juveniles, however, readily consumed fireflies and mealworms when placed in their home containers. There were no significant differences in

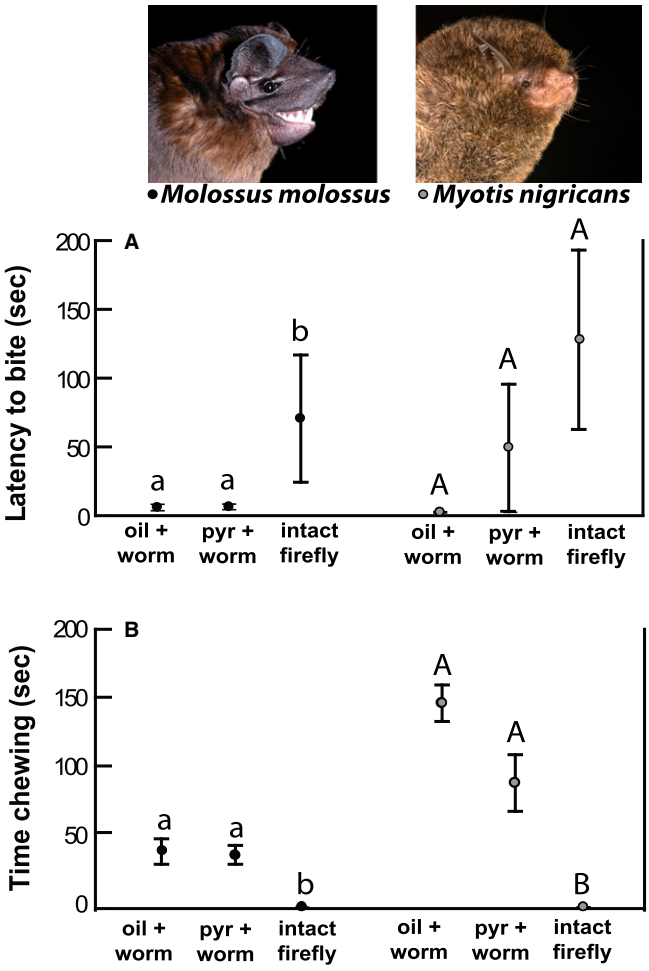

FIGURE 4. Responses of the tropical insectivorous bats, *Myotis nigricans* (N = 9) and *Molossus molossus* (N = 7) to test items presented in bioassays: (A) latency to bite; (B) time spent chewing individual prey items. Worm = *Tenebrio* mealworm. Pyr = pyrazine. Mean ± SE with the same letter are not significantly different. Photos: M. Tschapka.

the likelihood of consuming fireflies versus mealworms (Fisher's exact test: N = 22, P = 1.0).

Of the 25 adult toads that completed the trials, six individuals (25%) refused to feed in at least one of the trials presenting either a firefly or a pyrazine-treated mealworm. However, this trend was not sufficiently strong to suggest a specific rejection of intact fireflies or of pyrazine. While three of the six toads did not eat the firefly, only two of those individuals ate the last control mealworm, which indicates that the majority of toads remained motivated to feed. There was no significant effect of trial, firefly, or of individual tested (P = 0.613; P = 0.383; P = 0.062, respectively).

DISCUSSION

Fireflies were unpalatable to two of the three potential predators tested here. While cane toads readily consumed all offered prey types, *Azteca* ants and bats avoided intact fireflies. In contrast, ants readily attack both *P. trivittata* and *B. amoena* fireflies once the compounds surrounding them (odors or tastes) were experimentally removed. Firefly unpalatability is thought to be based on the noxious lucibufagens (LBGs), which become externalized during reflexive bleed when fireflies are disturbed (Meinwald *et al.* 1979). However, fireflies could be protected from predators by several additional lines of defense, which can include aposematic light warnings, such as glows or flashes, deterrent warning odors, and an unpalatable 'bad' taste.

There is substantial evidence that predators can associate visual and chemical signals with prey unpalatability and that the combination of such multi-modal warnings increases the effectiveness of aposematic defenses (Rowe & Guilford 1999, Mappes *et al.* 2005, Siddall & Mappes 2008). Several predators respond to firefly flashes and some are capable of associating them with food unpalatability. For example, the big brown bat, *Eptesicus fuscus,* is reluctant to attack flashing aerial lures (Vernon 1981), but the little brown bat, *Myotis lucifugus,* preferentially attacks flashing over non-flashing lures (Moosman *et al.* 2009). Long *et al.* (2012) demonstrated that jumping spiders (*Phidippus*) are able to associate noxious fireflies containing LBGs with a flashing LED. Nevertheless, the strong smell of a firefly when disturbed, as well as our results with ant and bat bioassays suggest that chemical signals, such as warning odors or taste, might also be an important line of defense for this group of insects.

Both firefly species release a complex and unique mixture of low molecular weight cuticular and volatile organic compounds, which, individually or in mixtures, could be used as early warning cues by ants and bats. We focused on 2-methoxy-3-(1-methylpropyl) pyrazine found in *P. trivittata's* volatile mixture as this family of compounds is well known for its ubiquitous, natural odorants found in a wide range of aposematically colored insects (models and mimics), as well as chemically defended plants (Moore *et al.* 1990, Dossey *et al.* 2009). Pyrazines are known to function as defenses against vertebrate predators (Rowe & Guilford 1999, Siddall & Marples 2011). In predatory insects, like ants, they are known to function as trail and alarm pheromones (Vander Meer *et al.* 2010). In our bioassays, ants, and to a lesser degree, bats, responded negatively to solvent-washed fireflies with topically applied pyrazine or to pyrazine-laced mealworms. There was a trend for reduced acceptance of prey augmented with pyrazine in *My. nigricans*, but not in *Mo. molossus* bats. However, this reluctance to accept pyrazine-enhanced baits was significantly weaker than their rejection of intact fireflies. These observations are consistent with the idea that pyrazine accounts, at least in part, for the rejection of intact *P. trivittata* fireflies. But they cannot explain the strong rejection of *P. trivittata* from which this compound has been experimentally removed by both bat species, or the rejection of *B. amoena* fireflies that lack pyrazine altogether by ants and bats. Thus, the pyrazine detected in tropical *P. trivittata*, represents a novel addition to the firefly chemical defense arsenal. However, other components of the firefly defensive phenotype may mediate avoidance of fireflies as prey.

Our results indicate that there are likely to be many additional volatile compounds that may function as aposematic signals and/or contribute to firefly repellence and deterrence. Some compounds may function in firefly species recognition rather than in defense. For example, both firefly species had distinctive cuticular hydrocarbons (CHCs) profiles. In Coleoptera, particularly the Cerambycidae, CHCs act as contact sex pheromones (Lacey *et al.* 2008). CHCs have also been implicated in sexual recognition in diurnal fireflies. Although nocturnal *Photinus* fireflies had low or undetectable CHC levels in both sexes, diurnal fireflies showed higher CHC levels (Ming & Lewis 2010). It is possible that the final stages of firefly courtships may be guided by CHCs that serve as additional pre-zygotic reproductive isolation mechanisms. Whether CHCs in our tropical fireflies function in either defense and/or courtship remains to be determined.

There is some evidence supporting the idea that the ethyl alcohol, ketones, and sequiterpenes observed in firefly volatile emissions could function as repellents or deterrents. For example, 2-ethyl hexanol, a branched, eight carbon alcohol, is moderately to severely irritating to human skin and eyes (Martin 2006). The ketone, 2-heptanone, is a constituent of the alarm pheromones released by many Neotropical dolichoderine species, including members of *Azteca*, as well as in honey bee mandibular glands (Hölldobler & Wilson 1990). In addition, 2-octanone imparts a bitter taste to humans (Papachristoforou *et al.* 2012). Sequiterpenes are diverse and common plant and fungi secondary compounds known for their bioactivity. For example, a caryophyllene, which was detected in *P. trivittata*, is recognized plant defense against herbivorous insects (*e.g.*, Wang *et al.* 2009). The methyl ester of the small chain fatty acid, 3-methyl-2-butanoic acid may also contribute to the pronounced and immediate rejections of fireflies by these bat species. Butanoic (butyric) acid has a biting taste and pungent, sour odor and its related esters, due to their high volatility, may be encountered by a predator well before it attacks (Prasad 1980). Its related esters, like the one detected here may likely be encountered by a predator well before it attacks and thus serve as an aposematic warning.

Like ants, both bat species that we examined strongly avoided eating intact fireflies that were unable to reflexively bleed. Both bat species ate significantly less of the proffered fireflies than of any other type of test presentation. High rejection and low consumption rates are consistent with a pattern of reduced trial time, wherein bats spent the least amount of time feeding on fireflies. Overall, fireflies were unacceptable bat prey.

Why did cane toads attack intact, live fireflies and pyrazine-laced, and control mealworms with such alacrity? The high acceptance of fireflies may be due to lack of experience with this prey type. Because toads forage on the ground in proximity to humans, often in areas with high artificial light levels, they may not encounter large numbers of fireflies. Although toads may find fireflies distasteful, they may require repeated exposure to learn an aversion to them. For instance, other anuran terrestrial predators, such as *Bufo bufo*, frequently encounter firefly larvae on the ground and readily learn to avoid this chemically defended life stage (De Cock & Matthysen 2003). Alternatively, cane toad acceptance of fireflies could be a consequence of a long history of co-evolutionary interaction that has afforded these toads physiological tolerance to the toxins present in sympatric fireflies. As noted, *Photuris* fireflies, due to their capacity to co-opt toxins from their firefly prey, usually members of the genus *Photinus*, can produce LBGs. LBGs are toxic to a wide variety of un-adapted predators. These are structurally and functionally related to other steroidal pyrone toxins, such as the well-known toad bufodienolides and the cardenolides of many plants (Eisner *et al.* 1978, Dobler *et al.* 2012, Zhen *et al.* 2012). In fact, the ingestion of *Photinus* fireflies containing LBGs by Australian bearded lizards (*Pogona*), a popular pet around the world, is often lethal, presumably because these lizards have not evolved tolerance to the LBGs found in these widespread Neotropical fireflies (Knight *et al.* 1999). Long-term feeding experiments limiting toads to a strict firefly diet might distinguish between these hypotheses.

None of these fireflies have lines of defense that appear to be effective against all predators. As our results show, toads readily eat fireflies while ants and bats avoid them. Moreover, *P. trivittata* pyrazines are evidently narrowly targeted against ants as deterrents or repellents. However, they also appear to affect the predatory behavior of at least one bat species. These conclusions are consistent with the idea that multiple enemies have exerted conflicting selection on firefly defenses. Facing intense enemy selection, fireflies have evolved elaborate, multi-trait defense arsenals.

Theory has long assumed that the selective advantage of a defense depends on its efficacy against a broad spectrum of enemies, which implies that predator selection is more diffuse than pairwise (*sensu* Futuyma & Slatkin 1983, Ehrlich & Raven 1964). In this scenario, a single enemy species, or functional guild, might be sufficiently abundant, damaging, and historically persistent that a broad-spectrum defensive trait aimed at such a major threat might suffice. Alternatively, we might expect multiple defensive traits to evolve if there are trade-offs in efficacy among defenses against several enemies with different modes of attack. It has been frequently proposed that biotic interactions, particularly those between prey and predator, are more numerous and diverse at lower latitudes (Paine 1966, Janzen 1970, see Schemske *et al.* 2009). If so, we predict that tropical fireflies will have narrowly targeted defenses and larger arsenals compared to their temperate counterparts. Our data suggest the possibility that tropical fireflies have evolved narrowly targeted defenses, which could have resulted from selective pressures imposed by a more diverse enemy community. However, more experimental studies are required that use a battery of ecologically relevant enemies to determine the effective spectrum of defensive traits in both temperate and tropical firefly species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

FIGURE S1. Identification and quantification of 2-methoxy-3-(1-methylpropyl) pyrazine emitted by the firefly *Photuris trivittata*.

TABLE S1. *Composition of cuticular compounds extracted from Photuris trivittata and Bicellonycha amoena*.

TABLE S2. *Major peaks of the EI mass spectra of unidentified compounds m/z (%) in Photuris trivittata and Bicellonycha amoena headspace.*

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