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Defensive chemistry of lycid beetles and of mimetic cerambycid beetles that feed on them

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Summary. Beetles of the family Lycidae have long been known to be chemically protected. We present evidence that North American species of the lycid genera *Calopteron* and *Lycus* are rejected by thrushes, wolf spiders, and orb-weaving spiders, and that they contain a systemic compound that could account, at least in part, for this unacceptability. This compound, a novel acetylenic acid that we named lycidic acid, proved actively deterrent in feeding tests with wolf spiders and coccinellid beetles. Species of *Lycus* commonly figure as models of mimetic associations. Among their mimics are species of the cerambycid beetle genus *Elytroleptus,* remarkable because they prey upon the model lycids. We postulated that by doing so *Elytroleptus* might incorporate the lycidic acid from their prey for their own defense. However, judging from analytical data, the beetles practice no such sequestration, explaining why they remain relatively palatable (in tests with wolf spiders) even after having fed on lycids. Chemical analyses also showed the lycids to contain pyrazines, such as were already known from other Lycidae, potent odorants that could serve in an aposematic capacity to forestall predatory attacks.

Key words. Acetylenic acid – lycidic acid – antifeedant – predation – mimicry – Coleoptera – Lycidae – Cerambycidae

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Introduction

Beetles of the family Lycidae, throughout their tropical and subtropical range, share many of the attributes one associates with distastefulness in insects. Lycids are sluggish, soft-bodied, slow-flying, and often aposematic, and as model elements in aggregations they commonly cooccur with mimics (Carpenter and Ford, 1933). Considerable data, from both field observation and predation tests, provide evidence that lycids are chemically protected (Marshall and Poulton, 1902; Carpenter, 1921; Jones, 1932; Darlington, 1938; Linsley et al., 1961). Actual analytical work, however, has been scant on lycids (Moore and Brown, 1981), and for New World species, was non-existent.

It was our intent, here, to look into the defensive chemistry of North American lycids. For such purpose we investigated lycids of two prominent genera, *Calopteron* (Fig. 1A, B) and *Lycus* (Fig. 1C, H; Fig. 2A, G, H), the former typically solitary, the latter commonly gregarious, which we were able to obtain in numbers. We confirmed, in tests with thrushes and spiders, that these lycids are indeed distasteful, and found that they contain a novel acetylenic compound, herein designated as lycidic acid, which we characterized, and which proved to be deterrent in predation tests with spiders and coccinellid beetles.

A second objective was to look into the chemical implications of a remarkable relationship prevailing between certain *Lycus* species and longhorn beetles (family Cerambycidae) of the genus *Elytroleptus. Elytroleptus* beetles have been shown to be mimetic of *Lycus* and to mingle with these in their aggregations (Linsley et al., 1961; Selander et al., 1963). Highly outnumbered by the model *Lycus*, and uncannily imitative of these, they are

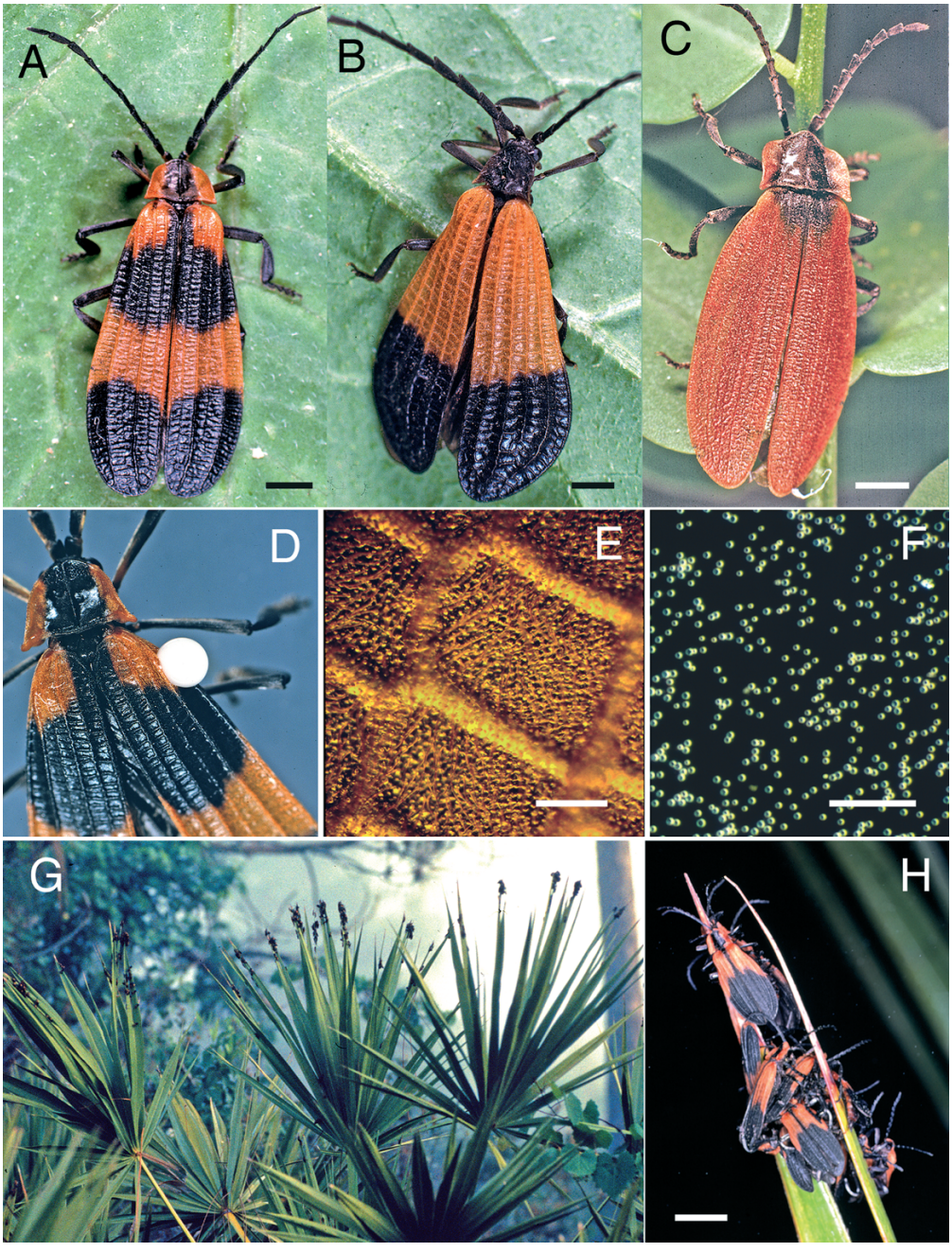


Fig. 1 (**A**) *Calopteron reticulatum;* (**B**) *Calopteron terminale;* (**C**) *Lycus sanguinipennis;* (**D**) *C. reticulatum,* bleeding from an elytron; (**E**) Detail of an elytron of *Lycus loripes,* showing the swollen veins, typical of lycids, from which blood is emitted when an elytron is injured; (**F**) Blood of *L. loripes*, at high magnification (dark field illumination), showing the minute (presumably lipoidal) droplets, that are a characteristic of lycid beetles; (**G**) An aggregation of *Lycus lateralis* on palmetto plants (*Serenoa repens*) in Florida (note that the lycids are grouped in clusters on the margin of the fronds; (**H**) Detail of preceding, showing one such cluster. (Reference bars: A—C = 2 mm; E = 100 μm; F = 10 μm; H = 5 mm)

generally hard to come by for study, but that is not what made them intriguing. *Elytroleptus,* in sharp contrast to cerambycids generally, which are phytophagous, had been found to be carnivorous (Eisner et al., 1962; Selander et al., 1963). They are highly selective in their dietary choice, and appear to feed mostly if not exclusively on the very *Lycus* they mimic. They attack these in their aggregations and consume a large portion of their bodies, raising the question whether in the process they appropriate the ingested lycidic acid for protective purposes of their own (Eisner et al., 1962). We report here on the isolation and characterization of lycidic acid from *Calopteron* and *Lycus*, as well as on the failure on the part of *Elytroleptus* to incorporate the acid from its *Lycus* prey. *Elytroleptus*, we show, albeit on the basis of scant data, are not rendered increasingly protected by consumption of *Lycus.* It was therefore to be expected that they would remain free of lycidic acid after ingestion of *Lycus*, which we were able to confirm.

Materials and Methods

Source and maintenance of lycids

The three species of *Calopteron* stemmed respectively from Ithaca, NY (*C. reticulatum*) (Fig. 1A); Madison, WI (*C. terminale*) (Fig. 1B); and Sebring, FL (*C. discrepans*) (not shown; very similar in appearance to *C. reticulatum*). Specimens were taken in woody areas, sitting on leaves of various kinds or slowly flying about.

The *Lycus* too were of sporadic distribution, but where found tended to occur in aggregations, sometimes by the hundreds. *Lycus lateralis,* the only species of the genus from the eastern U.S., was taken from an aggregation that had formed on saw palmetto (*Serenoa repens*) (Fig 1G, H), on the grounds of the Archbold Biological Station, Lake Placid, FL. The lycids had congregated on the margins of the fronds and were then highly conspicuous. The other *Lycus* [*L. fernandezi* (Fig. 2G)*; L. arizonensis* (Fig. 2H); *L. loripes* (Fig. 2A)*; L. sanguinipennis* (Fig. 1C); and *L. fulvellus* (not shown)], all stemmed from canyon country in southeastern Arizona, from locations in or near Portal, in the Chiricahua Mountains. They typically occurred on flowering plants (for instance, sweet white clover, *Melilotus alba*), densely clustered on the inflorescences.

In the laboratory we maintained lycids in groups, in plastic containers with miscellaneous floral cuttings (including *M. alba*), with access to water (cotton wad), under which conditions they survived for up to three weeks.

Source and maintenance of Elytroleptus

The *Elytroleptus* were of two species: (1) the concolorous *E. ignitus* (Fig. 2C) evenly orange-brown in coloration, a mimic of *L. loripes,* typically found in aggregations of the latter; and (2) E. *apicalis* (Fig. 2I), similarly orange-brown, but with black-tipped elytra, a mimic of *L. fernandezi,* typically found in aggregations of that particular lycid. It was these two species of *Elytroleptus* that had earlier been shown to be predators on *Lycus* (Eisner et al., 1962; Selander et al., 1963). Also previously noted was that *L. fernandezi* coexists with a second lycid in its aggregations, a lookalike with black-tipped elytra, *Lycus arizonensis* (Fig. 2H), that could potentially serve in a Müllerian capacity relative to *L. fernandezi* in the assemblages (Linsley et al., 1961). We found *L. arizonensis* to be present with *L. fernandezi* in some of our aggregation samples, and included the species in our analyses and bioassays. *Lycus loripes* also coexists with a congeneric lookalike in its aggregations, but that particular species, *L. simulans,* is of rare occurrence (Linsley et al. 1961), and appeared to be absent from the *L. loripes* assemblages sampled for the present study.

Elytroleptus, quite generally, occur in low numbers relative to their lycid models (Fig. 2B). Actual counts showed them to be outnumbered by lycids in the aggregations by a factor of 20 to 60 (Linsley et al., 1961). Their rarity, coupled with their lycid-likeness, and the fact that they tend to rest quiescent among the model lycids, makes them difficult to spot.

We kept the *Elytroleptus* individually in Petri dishes (9 cm diameter) on a "bedding" of floral cuttings (including *M. alba*). They were given water (cotton wad) and, in selected cases, lycids as prey. They were used in experiments within a few days following their arrival at Cornell by overnight mail from Arizona.

Palatability of lycids

Tests with thrushes. Seven birds (collected by mist-netting in Ithaca, NY) were available for testing – 4 hermit thrushes (*Hylocichla gutatta*) and 3 Swainson's thrushes (*H. ustulata*) – which were caged individually and offered a series of live lycids (*L. fernandezi*), in combination with edible controls in the form of live mealworms (larvae of the beetle *Tenebrio molitor*). The feeding protocol was basically that followed previously in palatability tests with fireflies (Eisner et al., 1978). The birds were tested in daily feeding sessions, in which they were given individual live *L. fernandezi* and mealworms, one at a time, in glass dishes. Sequence of presentation was such that each series of three consecutive items contained two mealworms and one randomly placed *L. fernandezi.* Each item was left with the bird until it was eaten or for a maximum of 3 min. Tests were continued for a given session until 5 of the lycids had been presented, or until the bird ceased responding to mealworms (which occurred in one case only, after presentation of the 4th lycid).

Bird responses were scored as follows: *eaten* (E, if the bird swallowed the item after pecking it no more than three times); *eaten with hesitation (*EH, if the bird ate the item after pecking it more than three times); *rejected* (R, if the bird ignored the item after pecking it one or more times); and ignored (I, if the bird failed to make contact with the item during the 3 min of presentation). The four hermit thrushes were each tested in a single daily session only (they received only male *L. fernandezi).* The three Swainson's thrushes were tested for 3 consecutive days each (two of them received only male *L. fernandezi*; the third only females).

Tests with wolf spiders. The tests with wolf spiders were also as previously described (Eisner and Eisner 1991). The spiders, *Lycosa ceratiola,* were collected on the grounds of the Archbold Biological Station, Lake Placid, FL, and maintained (either at Cornell or the Archbold Station) on mealworms and water, in individual cylindrical containers (16 cm diameter, 11 cm height) bearing a bottom layer of sand. The tests consisted simply of releasing individual lycids into the cages with the spiders and keeping track of events. Three species of lycids were tested: *L. loripes* (N = 21); L. *arizonensis* (N = 22); and *L. fernandezi* (N = 8). While the spiders were individually tested more than once, none was tested with more than one specimen per species. Nor were the spiders individually tested more often than once per day. For control purposes, to obtain a measure of the acceptability of a food item that we knew to be chemically unprotected, tests were done (N = 20) in which the spiders were offered individual mealworms.

A similar assay was carried out with another lycid, *L. lateralis*, but with the spider, *L. ceratiola,* in the wild, at its natural field site. The *L. lateralis,* had been taken from an aggregation they had formed on palmetto at the Archbold Station (Fig. 1G), and they were offered to *L. ceratiola* that were out at night on sandy terrain only meters from the lycid site, poised motionless on the ground in wait of prey. The spiders were located with headlamps by their eye shine, and they were fed by dropping individual lycids from vials onto the sand directly in front of them, causing them to pounce instantaneously upon the offering. Five *L. lateralis* were thus offered, to 5 individual spiders. Another ten *L. lateralis* (5 males, 5 females) were tested at the Archbold Station, again with *L. ceratiola* (10 individuals), but with caged rather than free-roaming spiders.

Tests with orb weavers. The spider in these tests was the familiar *Nephila clavipes,* with which we had experience (Eisner, 1982; Eisner et al., 1991), and which we tested outdoors, at a natural site where we knew it was abundant (Highlands Hammock State Park, Sebring, FL, some 40 km North of the Archbold Station). Tests involved flipping lycids singly from vials into the webs of individual spiders, and following events as the spiders then darted toward them from their resting position at the center of the orb. Lycids of two species were tested, *L. lateralis* and *C. discrepans,* both from Florida, where we knew them to co-occur with *N. clavipes* (the *C. discrepans* tested were in fact taken at Highlands Hammock).

Two *L. lateralis* were tested (both females), as well as 6 *C. discrepans* (all females). Five of the latter were offered, not just to a single spider, but sequentially to a series of *N. clavipes,* providing a basis for checking into both the durability of the lycid's noxiousness, and the variability in the spider's tolerance of that noxiousness.

Palatability of Elytroleptus

The question was whether ingestion of lycids conferred distastefulness upon *Elytroleptus*, and the answer was sought by testing for the acceptability of the cerambycids to wolf spiders. Tests were carried out with caged *L. ceratiola* (as described above for lycids) in which these were presented with *Elytroleptus* that had either been fed or been kept

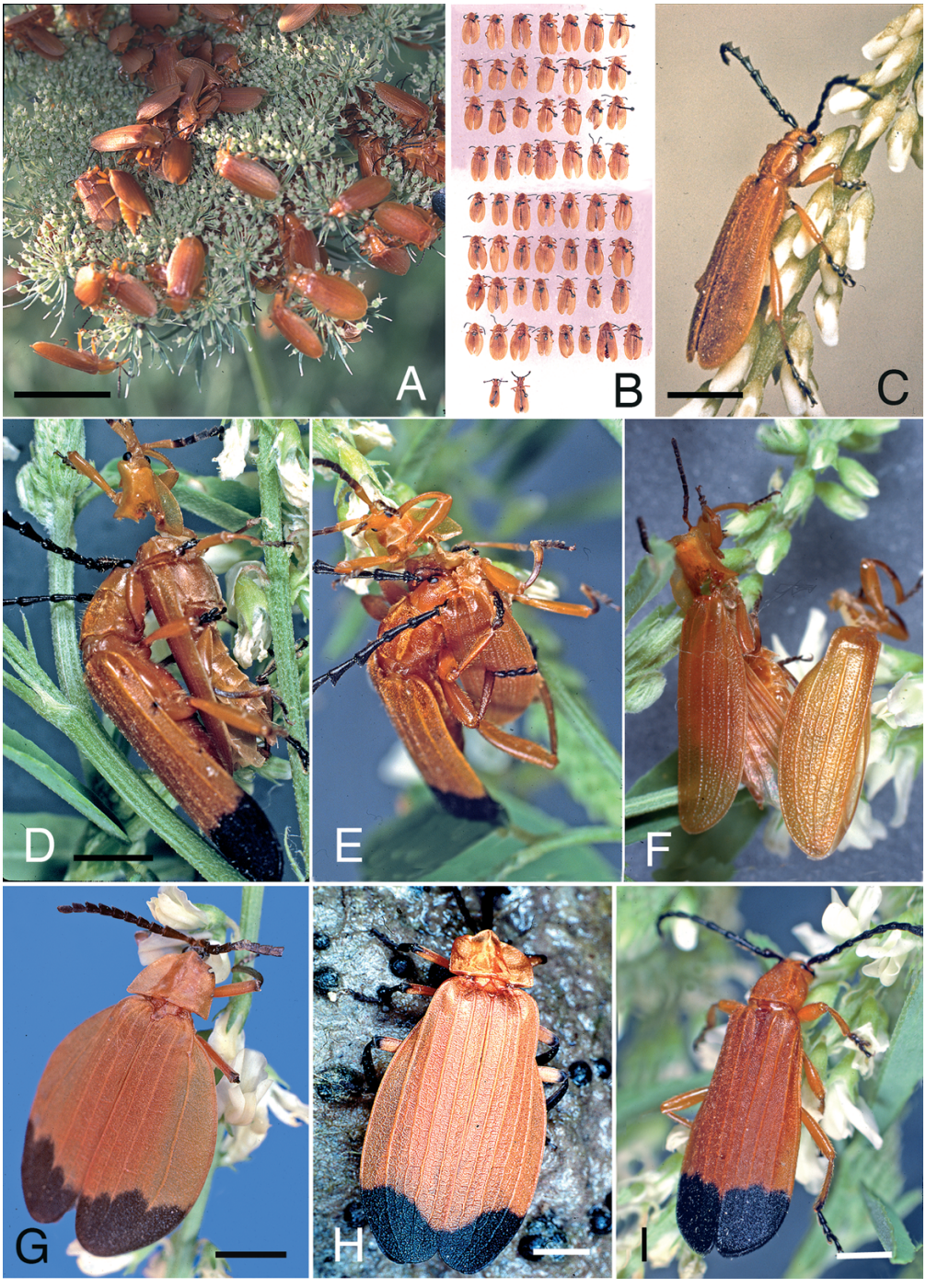


Fig. 2 **(A)** Detail of an aggregation of *Lycus loripes*, Arizona (photo by Noel Snyder). (**B**) Sample taken from one such aggregation, showing the skewed ratio of model to mimic. The latter, *Elytroleptus ignitus*, is represented by the two specimens in the bottom row. (**C**) *E. ignitus.* (**D-F**) Stages in the consumption of a lycid (*L. loripes*) by an *Elytroleptus* (the latter in this case is an *E. apicales*, a mimic not of *L. loripes,* but of *L. fernandezi*; in the laboratory *Elytroleptus* do not discriminate between these lycids. In (**D**) and (**E**) the cerambycid is chewing into the thorax of its victim; (**F**) shows the leftovers of the meal (it is quite typical for *Elytroleptus* to eat only a portion of its prey.) (**G-I**) The principal members of the *Lycus fernandezi* mimetic complex: (**G**) *L. fernandezi*; (**H**) *L. arizonensis;* (**I**) *Elytroleptus apicalis.* (Reference bars: A = 10 mm; C, D, G–I = 2 mm)

unfed on lycids. There was no way for us to determine the vulnerability of *Elytroleptus* at the time of pupal emergence, before the beetles had a chance to feed on lycids, because we did not know where to find the pupae. What we could do is determine whether the degree of defendedness of field-collected *Elytroleptus,* such as might already have fed on lycids and acquired a measure of the latter's distastefulness, is enhanced if such beetles are given a dietary supplement of lycids. Our hypothesis was that there should indeed be such enhancement and that the subsidized *Elytroleptus* would have a higher survival rate in the spider tests than the unsubsidized ones.

We had 15 *E. apicalis* available for testing and divided these into a group of 7 (the lycid-fed group) and a group of 8 (the lycid-unfed group). The 8 members of the unfed group were kept individually, isolated from lycids, during the period (2–4 days) intervening between their receipt from Arizona and their being tested with the spiders. The 7 members of the lycid-fed group were treated similarly, except that during the period prior to testing they were each confined with a number of lycids, some of which they attacked and ate (that is, partly consumed) (three ate 1 *L. fernandezi* each; one ate 2 *L. fernandezi;* one ate 1 *L. fernandezi* and *1 L. arizonensis;* one ate 1 *L. loripes*; and one ate 3 *L. loripes*).

The two *E. ignitus*, also slated for testing with spiders, were both confined with lycids, and partly ate 1 *L. loripes* each, prior to being offered to the spiders.

The *Elytroleptus* were scored as accepted or rejected, depending on whether they were eaten or released by the spiders.

Chemical Analyses of Lycids

Adult beetles of the following species were analyzed: *C. reticulatum* (~200); *C. terminale* (~100); *L. loripes* (~200); *L. fernandezi* (10); L. *arizonensis* (14); *L. sanguinipennis* (12); *L. fulvellus* (24). For chemical analysis, lycid beetles were freeze-dried, ground to a fine powder, and extracted with dichloromethane (2 ml per beetle). After filtration over cotton, the extracts were evaporated to dryness. The residue was redissolved in dichloromethane-d_2 and submitted to NMR-spectroscopic analysis, using a Varian INOVA 500 (500 MHz proton, 126 MHz carbon) spectrometer. Samples obtained from *L. loripes* and *C. reticulatum* were analyzed further via two-dimensional NMR-spectroscopy. Double-quantum filtered COSY (dqf-COSY) spectra were acquired using the standard Varian pulse sequence and phase cycling. Phase-sensitive NOESY spectra were acquired with a mixing time of 500 ms. Phase sensitive HMQC spectra and magnitude-mode HMBC spectra were acquired without gradients, using phase-cycling for coherence selection.

For isolation of pure lycidic acid, 80 adult *C. reticulatum* were extracted as described above. The resulting extract was chromatographed over silica, using hexane-ethyl acetate mixtures with increasing ethyl acetate content (15–70%) as solvent. Fractions containing lycidic acid were pooled and re-chromatographed, using a less polar solvent system (10–30% ethyl acetate in hexane). The resulting sample of lycidic acid (34 mg) was of greater than 95% purity, as determined by ^{1}H NMR spectroscopy. Impurities included small amounts of oleic acid and a dihydroderivative of lycidic acid. For two species, *L. loripes* and *C. reticulatum*, the amount of lycidic acid extracted per beetle was determined by comparison of NMR spectra obtained for crude extracts of *L. loripes* and *C. reticulatum* beetles with the NMR spectrum of an external standard prepared by dissolving 0.5 mg of 95% pure lycidic acid in 0.6 ml of dichloromethane-d_2.

NMR-spectroscopic data of lycidic acid (octadeca-5E, 7E-dien-9-ynoic acid): ^{1}H NMR (500 MHz, C_6D_6) δ [ppm] 0.89 (t, 3H, $J_{17,18}=7.3$ Hz, 18-H), 1.16–1.24 (m, 6H, 14-H, 15-H, 16-H), 1.26 (m, 2H, 17-H), 1.34 (m, 2H, 13-H), 1.41 (m, $J_{2,3}=7.6$ Hz, $J_{3,4}=7.2$ Hz, 2H, 3-H), 1.46 (quin., $J_{11,12}=J_{12,13}=7.1$ Hz, 2H, 12-H), 1.74 (m, $J_{4,5}=7.2$, 2H, 4-H), 1.96 (t, 2H, 2-H), 2.25 (dt, $J_{8,11}=2.1$ Hz, 2H, 11-H), 5.29 (m, $J_{5,6}=15.1$ Hz, 1H, 5-H), 5.62 (dt, $J_{7,8}=15.8$ Hz, 1H, 8-H), 5.82 (dd, $J_{6,7}=10.8$ Hz, 1H, 6-H), 6.61 (dd, 1H, 7-H); ^{13}C NMR (126 MHz, C_6D_6) δ [ppm] 14.30 (C-18), 19.94 (C-11), 23.02 (C-17), 24.11 (C-3), 31.94 (C-4), 29.22 (C-12 or C-13), 29.24 (C-12 or C-13), 29.48 (C-14 or C-15), 29.57 (C-14 or C-15), 32.18 (C-16), 33.29 (C-2), 80.71 (C-9), 92.94 (C-10), 111.00 (C-8), 131.16 (C-6), 134.92 (C-5), 140.83 (C-7), 180.29 (C-1).

High resolution mass spectra of isolated lycidic acid were acquired in GC-MS mode using a Micromass Autospec X mass spectrometer coupled to a Hewlett-Packard HP5890 gas chromatograph equipped with a 30 m, 0.25 mm i.d. DB5-MS column.

Chemical Analyses of Elytroleptus

Four specimens of *E. apicalis* and two of *E. ignitus* were separately analyzed for lycidic acid content. All had fed on *Lycus* beforehand (they were treated as were the lycid-fed individuals under "*Palatability of Elytroleptus"*, above). The four *E. apicalis* had eaten, respectively, 1 *L. loripes*, 2 *L. loripes,* 1 *L. fernandezi,* and 2 *L .fernandezi.* The two *E. ignitus* ate 2 *L. loripes* each. The *Elytroleptus* beetles were individually extracted as described above for the various species of *Lycus* and *Calopteron.* The resulting extracts were analyzed by NMR-spectroscopy and GC-MS.

Defensive Potency of Lycidic Acid

Tests with wolf spiders. The tests were similar to those described above (under the heading "*Palatability of lycids: tests with wolf spiders"*) except that they involved presentation of mealworms, which are highly acceptable to *L. ceratiola,* but can be rendered unacceptable if treated by topical addition of a noxious agent. We adopted a procedure whereby a spider was first given a mealworm and allowed to kill it with its cheliceral bite, and then, as it proceeded to feed on the prey, was tested for its response to having lycidic acid applied directly to its mouthparts.

The tests were straightforward. The spider typically grasped the mealworm the moment the latter was dropped in front of it, and proceeded immediately to inflict its bite. Without usually disengaging the chelicers it then commenced feeding, upon which (after about 3 min) we applied the test substance (a 1 μg/μL suspension of lycidic acid in glycerin). Application of the fluid was with a fine brush, pressed once into the cleft between the base of the chelicers. Application of glycerin itself served as control. A crude estimate of the quantity of sample delivered onto the spider by this procedure was obtained by determining the weight gain of a piece of glass (a "cover slip" such as is used in histology) comparably wetted by brushed application of glycerin. We found that we delivered something in the order of 10 μl of fluid in this fashion, amounting (in case of the experimentals) to about 10 μg of lycidic acid.

Tests and controls were repeated 18 times each.

Tests with coccinellid beetles. A second assay by which we tested for the deterrency of lycidic acid made use of a beetle, the coccinellid *Harmonia axyridis,* a predator that we found to feed eagerly on certain moth eggs, but to discriminate against these if they were treated by topical addition of a noxious substance. Paired presentation of egg batches treated and untreated by such addition can provide a basis for determination of chemical deterrencies. We used this assay to advantage previously and described it in detail elsewhere (Rossini et al. 2000).

For present purposes we used eggs of the moth *Utetheisa ornatrix* as food items. Ordinarily these eggs are unpalatable to predators on account of their contained pyrrolizidine alkaloids, derived from their natural parental diet. In the laboratory, however, the moth can be reared on a pyrrolizidine alkaloid-free diet, with the result that the eggs themselves are then alkaloid-free and palatable to *H. axyridis.*

Tests were done with individual adult *H. axyridis,* housed in Petri dishes (5 cm diameter). Ordinarily maintained in the laboratory on a diet of aphids, the beetles were starved for 2 days prior to experimentation, and given water only during this period. For experimental purposes they were each provided with two batches of *Utetheisa* eggs (10–12 eggs/batch), still affixed to the wax paper backing upon which they had been laid. One batch (experimental) was treated by topical addition of lycidic acid in methanolic solution, the other (control) was treated by application of methanol only. Solution or solvent was administered onto the egg clusters with a micropipette. Time was provided for evaporation of the methanol, prior to presentation of the two egg samples to the coccinellid. The batches were fastened by their wax paper backing to the Petri dish floor with double-sided sticky tape.

Lycidic acid was tested at two concentrations (1.0 and 5.0 μg/μL). The solutions were dribbled sequentially onto the eggs of a batch in fixed amounts (1 μL per egg), so that each egg of the batch received the same dosage of acid (1.0 or 5.0 μg). Control eggs received 1 μL methanol each.

Tests were of 2 h duration, with counts made at 15 min intervals of the number of eggs per batch remaining intact. Sample sizes for the 1.0 and 5.0 μg/egg tests were respectively $N=20$ and $N=24$. Different beetles were used for all trials.

For statistical purposes the data (proportion of eggs remaining intact) were subjected to arc sine transformation. Transformed data for each dosage and its control were subjected to two-way analysis of variance (ANOVA) with replication (Excel) (Snedecor and Cochran, 1989).

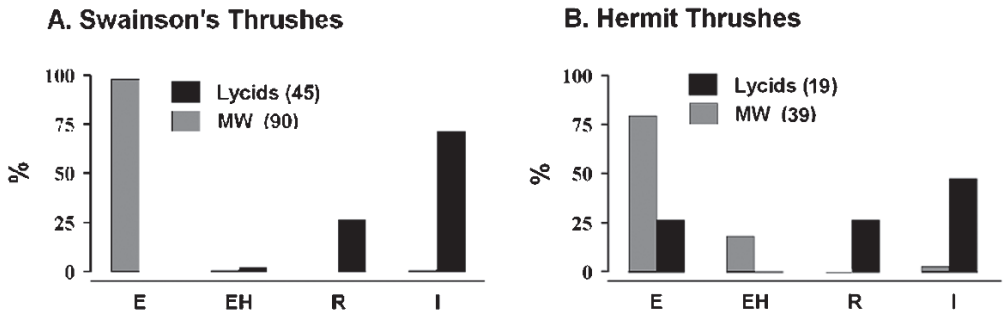


Fig. 3 Fate of lycids (*L. fernandezi*), relative to that of mealworms (MW) in tests with thrushes. Results are lumped for 3 Swainson's thrushes (**A**) and 4 Hermit thrushes (**B**). Fate of prey item is scored as eaten (E), eaten with hesitation (EH), rejected (R), and ignored (I). Details in text.

Results

Defensive hemorrhaging by lycids

As is well known to naturalists, lycids tend to bleed (that is, emit haemolymph) when disturbed (Fig. 1D). The phenomenon reveals itself readily, given that lycid blood is emitted in discrete droplets and is light-colored, ranging from white to pink. We noted blood emission to occur frequently when we handled our lycids in routine ways. Our observations confirm those by Darlington (1938), who provided an excellent description of the phenomenon. We noted, as did Darlington, that lycids can bleed from diverse body sites, as from between the abdominal segments, from sutures of the thorax, and from the tips of the legs. Most frequently, however, they bleed from the elytra, which possess swollen blood-filled veins that easily rupture (Fig. 1E). Surprisingly little pressure, applied to an elytron, will induce blood emission from an elytral vein.

As we point out in the sections that follow, we found blood emission to occur often in the course of the predatory attacks we staged upon lycids. The oozing of light-colored droplets was hard to miss when it occurred from beetle parts exposed to view.

Microscopic examination (under dark field illumination) (Fig. 1F) of blood droplets emitted in response to elytral pinching by both *Calopteron* (*C. reticulatum*) and *Lycus* (*L. fernandezi, L. loripes*) species, revealed the blood to contain a finely dispersed inner phase, in the form of minute, remarkably constant-sized spherules.

Palatability of lycids

Tests with thrushes. The results, lumped for the individuals of each species of thrush, and plotted separately for the two species, are shown in Fig 3A and B. Both sets of birds expressed a clear preference for mealworms over lycids (*L. fernandezi*) (chi square test: $p << 0.001$, $df = 3$). Mealworms generally were eaten outright, and in only few instances taken with hesitation, while the lycids were for the most part ignored. One lycid did get eaten by one of the Swainson's thrushes, but that individual was taken with hesitation and was the very first lycid of the 15 presented to that bird. With the hermit thrushes there was a higher incidence of lycid acceptance (5 of 19 offerings), but the acceptances here were all by a single bird which was anomalous in that it ate outright, without hesitation, all five lycids it was offered.

Lycids that were rejected, that is, pecked in the course of being inspected by the bird, had a high incidence of survival. Of the 12 lycids that were so treated by the Swainson's thrushes, 10 showed no signs of injury when examined the day following the attack.

Female lycids, available in lesser numbers and therefore tested with one Swainson's thrush only, fared no differently than the males.

Tests with wolf spiders. Not one of the total of 66 individuals of the four *Lycus* species tested, was accepted by *L. ceratiola* (Fig. 4A; note that for *L. lateralis* the bar incorporates the results of both the 5 field- and 10 laboratory-tests). The spiders typically pounced upon the lycids the moment they came into contact or near contact with them, but then, often within the second, backed away. The lycids took no evasive action when seized. Quite on the contrary, they seemed to "freeze" the moment they were grasped, as if programmed to anticipate release. The fate of the control mealworms was the exact opposite: not one of the 20 individuals offered was rejected by the spiders.

Bleeding, on the part of the lycids in these encounters did occur, but not, apparently, with each attack. Mere contact with the lycid, without induction of bleeding, appeared to suffice in some instances for spiders to discontinue their assault.

In these tests we did not keep track of the sex of the individual lycids (we sexed only the 10 indoor-tested *L. lateralis*). However, we had noted earlier that the lycid samples from which we took the series of *L. fernandezi, L. arizonensis,* and *L. loripes* that we fed to the spiders were of mixed sexes.

Tests with orb weavers. All 8 of the lycids tested (2 L. *lateralis* and 6 *C. discrepans),* upon being offered to individual *N. clavipes,* were rejected. The spider in each case converged upon the lycid the moment the latter was dropped in its web, and then, typically after no more than a brief inspection, set the beetle free. It either pulled the lycid from the web and let it drop, or cut it lose by using its chelicers in conjunction with legs and palps to sever the

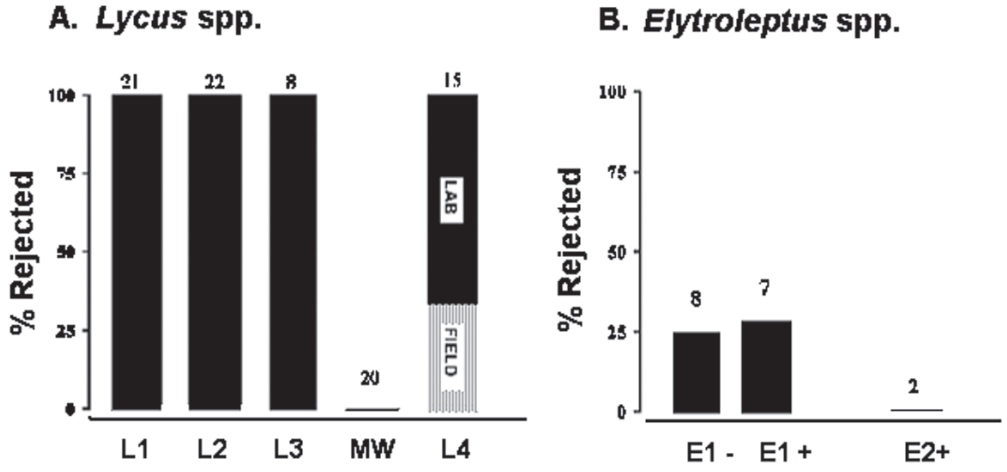


Fig. 4 (**A**) Fate of 4 species of *Lycus* (L1 = *L. loripes*; L2 = *L. arizonensis*; L3 = *L. fernandezi*; L4 = *L. lateralis*) and of mealworms (MW) in tests with the wolf spider, *Lycosa ceratiola.* For *L. lateralis,* the results of the 5 field- and 10 laboratory tests have been lumped. Numbers above columns give sample sizes. (**B**) Fate of *Elytroleptus apicalis* (E1) and *E. ignitus* (E2), in tests with the wolf spider, *Lycosa ceratiola.* Numbers above columns give sample sizes. The *Elytroleptus* are designated as (+) or (-) depending on whether they were given a supplement of lycid prey. Details in text.

strands that were imprisoning the beetle. It is quite typical for *N. clavipes* to rid itself of unwanted prey in this fashion (Eisner 1982).

Five of the 6 C. *discrepans* (all females) were re-tested on that same day or on subsequent days, with individual spiders that had not been used for testing on those days. Two individuals that were re-tested after an interval of 7 days, were found again to be rejected. Another individual, re-tested an additional 19 times on that first day, was rejected on each occasion by the spiders. A fourth individual, re-tested three more times on the first day, was rejected by the first two of these additional spiders, only to be eaten by the third. The fifth individual was rejected by a total of 11 spiders on day one, then again by 5 spiders on day 3, and finally on day 5 by another 7 spiders, before being eaten by the 8th. The two lycids that were eaten were thoroughly consumed, being reduced (in typical spider fashion) to compact packets of indigestible remnants.

Predatory behavior of Elytroleptus

The feeding of lycids to *Elytroleptus,* preparatory to the offering of the latter to wolf spiders, gave us the opportunity to observe in some detail the predatory behavior of these cerambycids (Fig. 2D–F). It was clear, first of all, that in executing their attack, *Elytoleptus* seemed to adhere to a protocol. As a rule the cerambycid eats only the central portion of the body of its victim, that is, the thorax, base of the abdomen, and parts of the legs and wings. It captures its victim by crawling upon it, usually from behind, and then straddling it from above so that it comes to embrace it with its legs. Chewing involves eating part of the thorax first, and then proceeding to the adjacent parts. Sometimes no more than an injury to the thorax is inflicted or to the base of the wings (in the latter case the lycid may survive), but in the laboratory, at least, such minimal sampling of a *Lycus* was the exception. Blood, such as seeped visibly from the lycid's wounds, appeared to be largely imbibed by the cerambycid. Given that the cerambycid is of about the same size as its lycid prey, and that in the aggregations the lycids are plentiful, it came as no surprise that that *Elytroleptus* were essentially "sloppy" eaters that consumed lycids only in part.

Quite remarkable is the fact that the lycid makes no physical effort to rid itself of its attacker, even in the early stages of the assault while it is still live and (one would think) able to take evasive action. In fact, as was clear from observation of *Elytroleptus* confined with lycids in Petri dishes, the lycids appear to be totally oblivious to the presence of the predator in their midst. The cerambycid in turn appears to be programmed not to "stir things up." Both in the laboratory and in the field, when moving about among lycids, the *Elytroleptus* were noted to do so at a deliberate, leisurely pace.

Somewhat surprising is the fact that neither of the two *Elytroleptus* species tested appears to be rigorously prey specific. They both seemed as ready to consume *Lycus* that were not in their own image, as those that they mimicked. Such lack of absolute prey specificity had previously been noted (Eisner et al., 1962, Selander et al., 1963).

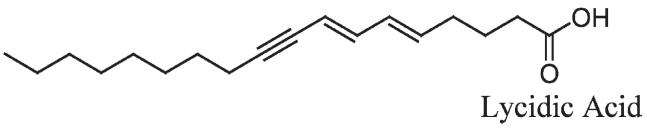
Palatability of Elytroleptus

Contrary to expectation, both species of *Elytroleptus* proved relatively acceptable: fully 11 of the 15 *E apicalis* and 2 of the 2 *E. ignitus* that were tested were eaten by the spiders (Fig 4B). Their fate was evidently very different from that of the lycids (Fig 4A) in the same assay (chi square test: $p < 0.001$, $df = 1$, for the comparison). Having received a supplement of lycids appeared to have no effect on the palatability of the cerambycid: *E. apicalis* that

received the supplement stood the same chance of being eaten as those that were kept unfed (chi square test: $p > 0.85$, $df = 1$, for the comparison between E1+ and E1–). The data were evidently incompatible with the notion that the vulnerability of *Elytroleptus* was a function of the number of lycids eaten.

Chemistry of lycids

The ^{1}H NMR spectra of extracts from all seven species of lycid beetles revealed the presence of large quantities of mixtures of unsaturated fatty acids. Analysis of two-dimensional NMR spectra obtained for crude extracts of *C. reticulatum* and *L. loripes* indicated that a highly unsaturated fatty acid featuring an yne-diene motif constituted the major component of the fatty acid mixtures in these species. Subsequent comparison of the ^{1}H-NMR spectra obtained for the other five species of lycid beetles with those obtained for *C. reticulatum* and *L. loripes* indicated that this unusual fatty acid represents a major component in extracts of beetles from all lycid species included in this study. A two-step chromatographic fractionation of the extract obtained from a large number of *C. reticulatum* beetles yielded a pure sample of the ynedienoic acid. Analysis of the isolated sample via two-dimensional NMR spectroscopy indicated a straight-chain fatty acid featuring a triple bond in position 9 and double bonds in positions 5 and 7. High-resolution mass spectrometry revealed a molecular ion at m/z 276.2078 corresponding to $C_{18}H_{28}O_2$ (calculated: m/z 276.2089). In conjunction with the results from the NMR-spectroscopic analyses, these MS data determined the structure of the major component of the lycid beetle extracts as octadeca-5E, 7E-dien-9-ynoic acid, which we named lycidic acid.



Lycidic Acid

Beetles from all seven species contained large amounts of lycidic acid. NMR-spectroscopic analyses of individual *L. loripes* and *C. reticulatum* beetles revealed between 0.2 and 0.8 mg of extractable lycidic acid per beetle.

In addition, GC-MS analyses revealed presence of 2-methoxy-3-isopropylpyrazine in the whole body extracts of all lycid species investigated herein. This odorous factor had been isolated previously from an Australian lycid (Moore and Brown, 1981; Moore et al., 1990). Smaller amounts of structurally similar pyrazines were also detected in our extracts but were not characterized further.

Chemistry of Elytroleptus

None of the *Elytroleptus* extracts contained lycidic acid, as determined by analysis via NMR spectroscopy and GC-MS.

Defensive potency of lycidic acid

Tests with wolf spiders. Of the 18 spiders that were stimulated with lycidic acid, 15 responded by releasing their hold on the mealworm they were eating (Fig. 5). Only 3 continued feeding, although we noted that these did so after shifting their mouthparts to a new feeding site, in response possibly to the original site having become contaminated with the test substance. Control stimulation with glycerol had no effect on the spiders, which reduced the mealworms they were eating to small packets of remains (chi square test: $p << 0.001$, $df=1$, for the comparison of experimentals and controls).

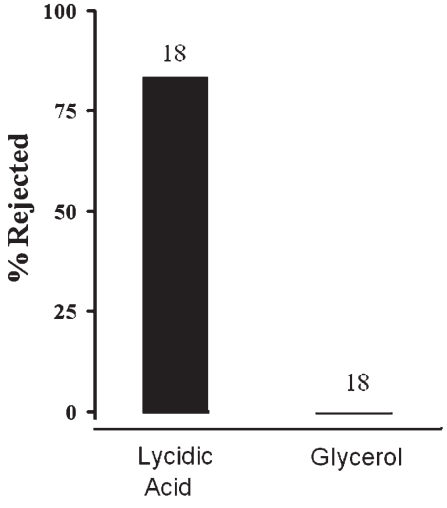


Fig. 5 Response of wolf spiders (*Lycosa ceratiola*) to oral administration of lycidic acid. The spiders had been feeding on mealworms when the acid (at an approximate dosage of 10 μg in 10 μL of glycerol) was applied to their mouthparts. The per cent spiders is here plotted that abandoned the mealworm when thus stimulated. The reaction to glycerol (10 μL) provides the control. The difference in spider response to experimentals and controls was significant (chi square test: $p \ll 0.001$, $df = 1$)

Tests with coccinellid beetles. As is evident from Fig. 6, lycidic acid proved deterrent at both dosages tested. The increased consumption rate of the control eggs over that of the experimentals was highly significant at the 5.0 μg/egg dosage ($p < 0.001$) and moderately significant at the 1.0 μg/egg dosage ($p < 0.05$).

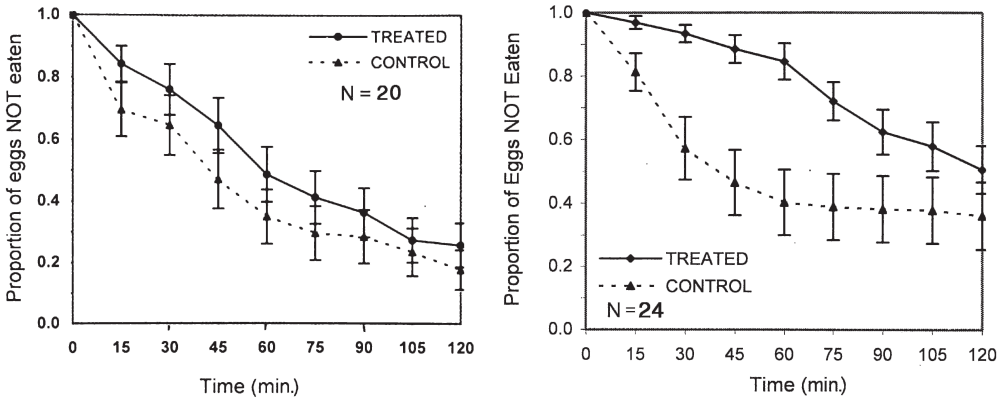


Fig. 6 Feeding response of individual coccinellid beetles (*Harmonia axyridis*) to paired presentation of treated and untreated moth egg batches. Treated eggs received a topical dosage of lycidic acid, administered in methanolic solution; untreated eggs (controls) received methanol only. Data give the number of eggs per treated and control batches remaining uneaten, as a function of time. Lycidic acid was tested at two dosages: 1.0 μg/egg (left) and 5.0 μg/egg (right). Details in text.

Discussion

Our results are evidently supportive of the view that lycids are unacceptable to predators. Classic studies, such as those of Jones (1932), had demonstrated that the North American lycid, *Calopteron reticulatum,* is generally shunned by birds when offered freshly-killed on feeding trays outdoors in combination with other insects, and Darlington (1938), working with West Indian lycids of the genus *Thonalmus* showed these to be unacceptable to *Anolis* lizards. Unacceptability of lycids to lizards was also demonstrated by Selander et al. (1963). Additional experiments with baboons and a kestrel from Mashonaland (Marshall and Poulton, 1902), and with African *Cercopithecus* monkeys (Carpenter 1921), showed these predators also to discriminate against lycids. Our finding that our thrushes proved aversive to lycids therefore came as no surprise. Birds may quite generally discriminate against lycids. As pointed out by Jones (1932), C. *reticulatum,* the very lycid he himself had found to be shunned by birds, does not appear in the listing of 337,000 insects identified from the stomach contents of some 80,000 birds, tabulated by W. L. McAtee (1932).

Little data existed on the acceptability of lycids to invertebrates, although such information as was available was telling. Darlington (1938) reports seeing a robber fly (family Asilidae) promptly release a lycid that it caught in mid air, and Linsely et al. (1961) noted a *Polistes* wasp to release an individual *Lycus* (probably *L. loripes*) that it had captured and inspected. There was also some evidence that lycids are unacceptable to ants, and less than fully acceptable to preying mantids (Linsley et al., 1961). Our finding that lycids are unacceptable to both a wolf spider and an orb weaver adds to this data, and is significant inasmuch as spiders, and orb weavers in particular, may figure prominently among the natural enemies of lycids.

A point worth noting is that, while effective, a lycid's defenses are not without limits. Serial introduction of individual *C. reticulatum* into N. *clavipes* webs revealed that the aversive properties of a lycid do not "wear off" in consequence of a single exposure to an orb weaver. The lycids all survived second exposures to *N. clavipes*, and in one case exposure to as many as 20 consecutive spiders in a single day. Whatever loss is incurred by a lycid in response to a single orb weaver attack – a droplet or two of blood, perhaps – is insufficient in itself to exhaust the lycid's defensive reserves. However, two of the lycids offered to the spiders did eventually get eaten. Both had earlier fended off spiders and might in consequence – perhaps by having bled too much – have become vulnerable. But it is also possible that in their final confrontation, the lycids had come upon spiders that differed from the norm, which on account of being either exceptionally hungry, or insensitive to the lycid's defenses, had been driven or enabled to press their assault. The latter alternative is worth pondering. Predators are bound to be variously sensitive to the weaponry of prey, and although we rarely have a grasp of the extent of this variability, there can be little question that it must factor into the subtleties of predator-prey interaction. Interestingly, judging from our data with thrushes, lycid-tolerance can be the mark of an occasional bird as well (witness the finding, that amongst our thrushes there was one that ate all lycids it was offered.)

The presence in lycid blood of a dispersed, seemingly lipoidal inner phase could account for why lycid blood is white or whitish, as oil emulsions typically are. One is tempted to suggest, that the tiny droplets that make up that inner phase of lycid blood are in fact lipoidal, and the carriers of lycidic acid (we should obviously have analyzed lycid blood for presence of lycidic acid, but failed to do so).

The finding that all seven *Calopteron* and *Lycus* species that we investigated chemically contain large quantities of lycidic acid was unexpected. Smaller quantities of similar acetylenic acids had been identified earlier from the one lycid previously studied, the Australian *Metriorrynchus rhipidius* (Moore and Brown, 1981). However, in *M. rhipidius*, a complex mixture of acetylenic acids was found, the acetylenic acids were not among the major lipids present in the beetle, and the total amounts of acetylenic acids per beetle were much smaller (in the order of 60 μg per beetle) and highly variable. Among

insects, the identification of 1-hydroxypentacosa-13E,15E,18Z,20Z-tetraen-11-yn-4-one 1-acetate from *Crematogaster* ants represents the only other example of a lipid with a similar oligoene-yne feature (Daloze et al., 1998). Interestingly, very similar dyenynoic acids are known from plants. For example, a closely related structural isomer of lycidic acid, octadeca-11Z,13Z-dien-9-ynoic acid, as well as several other related fatty acids have been identified from *Ximenia americana* (Hatt et al., 1960; Majekodunmi et al., 2000). Given that we found consistently large quantities of lycidic acid in all species of *Calopteron* and *Lycus* we had available for study, and because the quantities of lycidic acid contained in beetles from different locales and collection times showed little variation, it seems unlikely that the beetles sequestered the compound from plants. However, little is known about the life cycle of lycids, let alone their larval diet, and thus we cannot exclude the possibility that both *Lycus* and *Calopteron* obtain lycidic acid from acetylenic fatty acid-producing plants.

As reported already for *M. rhipidius* (Moore and Browne, 1981; Moore et al., 1990), and as we noted to hold true for all species studied by us, lycid beetles have a faint but distinct quinoline-like odor, attributed apparently to its pyrazines, of which 2-methoxy-3-isopropylpyrazine may be the chief compound shared by all. The question remains open whether such pyrazines have a defensive function, and if they do, how it is expressed. A current view, which we share, is that the pyrazines, rather than acting directly as repellents, play an aposematic function, that is, a warning function, by which the predator is alerted to the noxiousness of lycid prey. "Desist, lest you are willing to risk an unpleasant mouthful," may be the message implicit in the emitted pyrazines, and predators might well take heed. The general topic of chemical aposematism in insects has received considerable attention over the years (Eisner and Grant, 1981; Guilford et al., 1987; Kaye et al., 1989; Milhara at al., 1991; Wolfson and Rothschild, 1990). In lycids, one could well imagine the pyrazines acting as chemical re-inforcers of the visual aposematism already achieved by lycids through their gaudy coloration.

And finally, there are our findings with *Elytroleptus.* We fully expected these to be rejected by the jumping spiders, certainly after their laboratory feedings on lycids, but this was not to be. *Elytroleptus* proved persistently palatable even after having been fed lycids, and they showed no systemic build-up of lycidic acid in consequence of such feedings. Perhaps it was the cerambycid's *non*-donning of the defensive "mantle" of their lycid prey that should have been expeccted. Previous data, although scant, pointed to the acceptability of *Elytroleptus* to grasshopper mice and to a praying mantid (Linsley at al., 1963), as well as to lizards (Selander et al., 1963). The vulnerability to these particular predators, like that to jumping spiders, might well derive from the cerambycid's failure to make secondary use of lycidic acid. How *Elytroleptus* goes about inactivating the acid remains a mystery, although one could envision such inactivation proceeding quickly, as the cerambycids eat the lycids, by way of standard (or especially modified) fatty acid metabolism.

The low ratio at which *Elytroleptus* occur relative to the lycids in the aggregations itself suggested that they might be edible elements of their associations. Being, so to speak, Batesian in character, they might have been "forced" evolutionarily to maintain a low profile. Had they themselves been inedible (that is Müllerian), they might perhaps have occurred in larger numbers relative to the lycids, although one could equally argue that their being predacious on the lycids would impose intrinsic constraints upon their numerical representation in the aggregations, quite irrespective of palatability.

We are reluctant to speculate extensively on the observation that *Elytroleptus* are not rigorously specific in their choice of lycid prey. In our laboratory setting, both *E. apicalis* and *E. ignitus,* seemed ready to feed on any *Lycus* they were offered. It would be interesting to know whether lycidic acid itself plays a role in prey recognition in *Elytroleptus*, and whether in consequence of the seeming ubiquitous presence of the compound in North American lycids, these lycids all taste alike to the cerambycids. Are the *Lycus* all equally attractive to the *Elytroleptus*, and do the latter essentially ignore the lycid's visual appearance when they select these for food? What, in fact, binds *Elytroleptus* to the aggregations of their model lycids, and how rigid are these attachments? How common are the incidences of *E. apicalis* feeding on the "wrong" model (that is on a concolorous *Lycus* instead of a black-tipped one) and could the detailed imitations of *Lycus* by *Elytroleptus* have evolved at all if such wrong pairings were the norm? These are questions that might be well worth pursuing in future work on these remarkable beetles.

Acknowledgements

This paper is dedicated in memoriam to David Utterback, who provided many of the *Lycus* used in the study, and whose untimely death deprived the resident community in Portal, Arizona of one of its most devoted naturalists. The study was supported by Grant AI 02908 from the National Institutes of Health. We are grateful to the staff of the Archbold Biological Station, Lake Placid, FL, for countless favors; to Dr. Robert Smith, of the Department of Entomology, University of Arizona, Tucson, for providing the *L. fernandezi* used in the tests with thrushes; and to Dr. D. K. Young, of the Department of Entomology, University of Wisconsin, Madison, for sending the *C. terminale.* This is paper no. 196 in the series *Defense Mechanisms of Arthropods*; paper no. 195 is del Campo et al., *Chemoecology* 17: 19–22 (2006).

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