DEFENSIVE PRODUCTION OF QUINOLINE BY A PHASMID INSECT (*OREOPHOETES PERUANA*)

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Summary

Adults and nymphs of the Peruvian stick insect *Oreophoetes peruana* (order Phasmatodea) have a pair of thoracic glands from which they discharge a malodorous fluid when disturbed. The secretion contains a single volatile component, quinoline. Quinoline has not been reported previously from an animal source. The compound proved repellent or topically irritant in assays with ants, spiders, cockroaches and frogs. *O. peruana* nymphs, at molting, do not extricate the shed cuticular lining of the glands, thereby managing not to lose their secretory supply when they cast their skin. They are able, as a consequence, to discharge secretion even while still teneral after molting.

Key words: chemical defense, predation, alkaloid, molting, *Oreophoetes peruana*, stick insect.

Introduction

Insects of the order Phasmatodea, the so-called stick insects, comprise some 2500 species, mostly from the warmer parts of the world (Bedford, 1978; Key, 1991). Large and frequently apterous, phasmids are commonly slow and clumsy and, for that reason alone, are vulnerable. Many, however, are cryptically colored, armed with spines or protected by resemblance to twigs and leaves. Others, comprising only a few known species, are protected by defensive glands. We recently studied a strikingly colored species, the Peruvian fire stick *Oreophoetes peruana* (Floyd, 1993) (Fig. 1A) which, when disturbed, ejects a white malodorous fluid from two glands in the prothorax (Fig. 1B). In the present study, we investigated the biology and chemistry of this defense mechanism and present our findings here.

Materials and methods

Oreophoetes peruana

The individuals of *Oreophoetes peruana* (Saussure) we studied were from a small colony maintained in the Cincinnati Zoo, OH, USA. Founding members of the colony had been collected by one of us (R.C.M.) approximately 70 km northeast of Iquitos, Perú, where they were noted to feed on ferns. Captive individuals, which fed readily on a number of temperate and tropical ferns, were maintained routinely on one species, *Nephrolepis exaltata* (Boston fern). The animals mated, oviposited and coexisted at all developmental stages on this plant. Females dropped their eggs singly, from their perches. Nymphs did not seek shelter to molt.

Their body coloration renders all stages of *O. peruana* highly conspicuous. Nymphs are black, with yellow and green stripes, and orange markings on their head and legs. Adult females retain that overall pattern, while males are predominantly black with red bands and markings. The males are thinner and somewhat shorter (6 cm) than the females (7 cm).

Chemistry

O. peruana were 'milked' of secretion by seizing individuals by hand and taking up the fluid that oozed from their glands into capillary tubes. Separate secretion samples (one sample from each group) were collected from females, males, and first-, second- and third-instar nymphs. Each sample contained secretion from several individuals.

Samples were extracted with dichloromethane and analyzed using gas chromatography (GC), gas chromatography/mass spectrometry (GC-MS) and gas chromatography/infrared spectroscopy (GC-FTIR). Gas chromatography was performed on an HP 5890 instrument using a 30 m×0.22 mm fused-silica column coated with DB-5 (J&W Scientific). The oven temperature was kept at 60 °C for 4 min, raised at 8 °C min^{-1} to 250 °C, and held at this temperature for 5 min. Electron-impact mass spectra were obtained on an HP 5890 gas chromatograph linked to an HP 5970 mass-selective detector (MSD) operated at 70 eV, using a 25 m×0.22 mm fused-silica column coated with DB-5. The oven temperature was kept at 60 °C for 4 min, and raised at 15 °C min^{-1} to 270 °C. GC-FTIR

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analyses were performed on an HP 5890 gas chromatograph coupled to an HP 5965A IRD instrument equipped with a liquid-nitrogen-cooled narrow-band (4000–750 cm^{-1}) infrared detector (mercury cadmium telluride). Spectra were obtained at 8 cm^{-1} resolution. A DB-5 column (25 m×0.33 mm fused silica; 60 °C for 3 min, increased at 10 °C min^{-1} to 250 °C) was used for the analyses. Samples were introduced by splitless injection (220 °C; purge delay 0.5 min). Reference spectra were collected (4–7 scans) immediately before or after a GC peak.

Quinoline, isoquinoline and naphthalene were obtained from Aldrich Chemical Co. Quinoline is a yellowish oily fluid of relatively high boiling point (238 °C).

The glands

Anatomical observations were made on individuals preserved in ethanol after they died. Since live individuals were needed for colony maintenance and secretion production, none was killed specifically for the purpose of cytological fixation.

Bioassays

Quinoline, in these assays, was used in pure form.

Ants

The tests with ants (*Monomorium pharaonis*) were carried out at the Archbold Biological Station, Lake Placid, Florida, USA, at a site where workers from a natural colony had laid foraging trails. Baits were presented to these ants, in the form of droplets of sugary fluid (dilute honey solution), placed beside the trails on circular glass coverslips (18 mm in diameter). Individual ants that had come to feed at the margin of the droplets were stimulated with either quinoline or water (control). The fluids were presented in capillary tubes (0.25 mm^2 bore), hand-held with the tip positioned 1–2 mm above the head of the ant. Ants that ceased feeding and backed away within 5 s of tube presentation (as opposed to those that continued to feed) were scored as respondents. Sample size was 100 ants per category.

Spiders

The spiders (*Lycosa ceratiola*), tested in cages, were also from the Archbold Station. Individuals were given a mealworm (larva of *Tenebrio molitor*) which they promptly took as prey. After they had commenced feeding, each was stimulated with either quinoline or water (control), applied as a droplet (0.3 μl) with a micropipette, directly to the base of the chelicers. Individuals that extricated the chelicers and abandoned the mealworm within 60 s were scored as respondents. Sample size was 13 spiders per category.

Cockroaches

The test with cockroaches was based on the observation that, when a droplet of irritant chemical is placed on one side or the other of the fifth abdominal tergite of a decapitated nymph of the cockroach *Periplaneta americana*, the animal scratches the site with the hindleg of the side stimulated. The time interval between the application of sample and scratching provides a measure of the irritant effectiveness of the chemical. Details of this assay, which has been used for assessment of irritancy of a number of natural products, are given elsewhere (Eisner *et al.* 1976). We used the assay to test for the potency of quinoline and *O. peruana* secretion, relative to water as a control. Droplets (0.3 μl) were applied with a micropipette and the delay to the onset of scratching was timed. Cockroaches that failed to scratch within 30 s were scored as non-respondents. The *O. peruana* secretion had been obtained from the phasmids a few days earlier and had been kept refrigerated.

Quinoline was also tested as a near-contact stimulus. To this end, the tip of a capillary tube (0.03 mm^2 bore) bearing quinoline was held 0.5–1 mm from the surface of the cockroach (to one side of the fifth abdominal tergite), and whether or not scratching occurred within 30 s was again scored. Sample size was 10 cockroaches per category.

Frogs

The frogs (*Pseudacris crucifer*, from Ithaca, NY, USA) were also subjected to a topical irritancy test. While resting, each frog was first stimulated by application of a droplet of water (0.2 μl) to one flank of the abdomen, then by application of a comparable droplet of quinoline to the opposite flank. The interval between the two applications was at least 2 min. Failure to scratch the site stimulated within 30 s of droplet delivery was recorded as no response. Five frogs were tested.

Results
The defense

Adults and all nymphal instars responded to disturbance by emitting secretion. The fluid was sometimes ejected as a spray (particularly by adult females, but also by nymphs at times), but most often it simply oozed forth as a pair of white, sizeable droplets from the margins of the prothorax, just behind the head (Fig. 1B). The droplets often merged and spread over the thorax. Even newly eclosed first-instar nymphs discharged visible quantities of secretion. As a rule, the insects responded only to direct contact stimulation. Mere movement nearby, or the tapping of their foodplant, tended not to cause them to discharge. The insects proved able to discharge even within minutes after molting, before their bodies had hardened. Inability to discharge occurred only during molting (only 1 of 15 individuals stimulated while molting produced droplets).

Chemistry

Gas chromatograms of the extracts of secretion from the three nymphal stages and from adults of both sexes all showed essentially one peak. The mass spectrum corresponding to this peak (Fig. 2A) was congruent with that reported for quinoline (McLafferty and Stauffer, 1989). However, since isoquinoline has a very similar mass spectrum (McLafferty and Stauffer, 1989), this single criterion is not definitive. Fortunately, the gas-phase infrared spectra of these isomeric heterocycles are significantly different (Pouchert, 1989), and GC-FTIR

Fig. 1. *Oreophoetes peruana*. (A) Male astride the female. (B) Front end of female, shortly after it has discharged a defensive secretion, showing the residue of the white secretion on the pronotum.
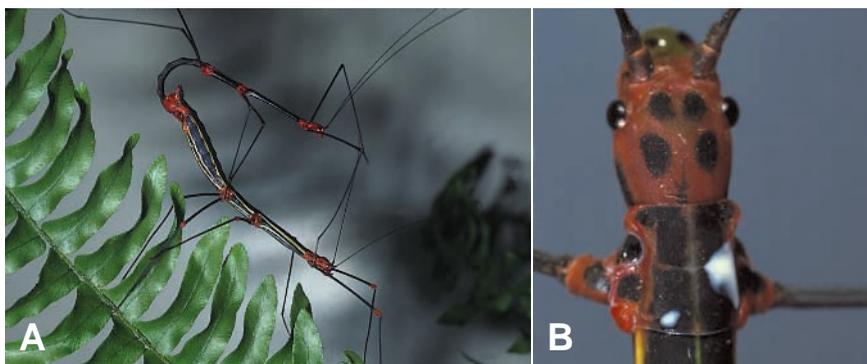
analysis (Fig. 2B) of the extract showed the single volatile component to be quinoline. This identification was confirmed by a direct comparison of the GC retention times of authentic samples of quinoline, isoquinoline and of the insect-derived material (Fig. 3).

Examination of the secretion under high magnification (200×) showed it to consist of a fine emulsion. We presume the inner phase to be quinoline (which is immiscible with water) and the outer phase to be water itself. Indeed, placement of a droplet of fresh secretion onto dried filter paper impregnated with cobatous chloride caused the paper to change color from blue to pink, indicating the presence of water.

Discharged secretion droplets, on exposure to air, undergo what we presume to be evaporative loss of water, as they lose their white coloration and spread to form thin oily films (presumably of quinoline). Body areas of the stick insect wetted by secretion remain visibly contaminated by such oily residue for minutes after an ejection. During this period, the animal reeks unmistakably of quinoline. The odor also tends to linger on the fingers after one has handled *O. peruana*.

To obtain some measure of the relative ratio of the two phases in the secretion, a single weighed sample of secretion (the pooled discharge from several females and nymphs) was analyzed by GC using naphthalene as an internal standard. Quinoline was found to make up 7 % (by mass) of the mixture. An emulsion, made artificially of quinoline (7 %) and water, was found to be white, like the secretion.

GC-MS analysis of a dichloromethane extract of crushed fronds of the fern (*N. exaltata*) upon which we maintained *O.*
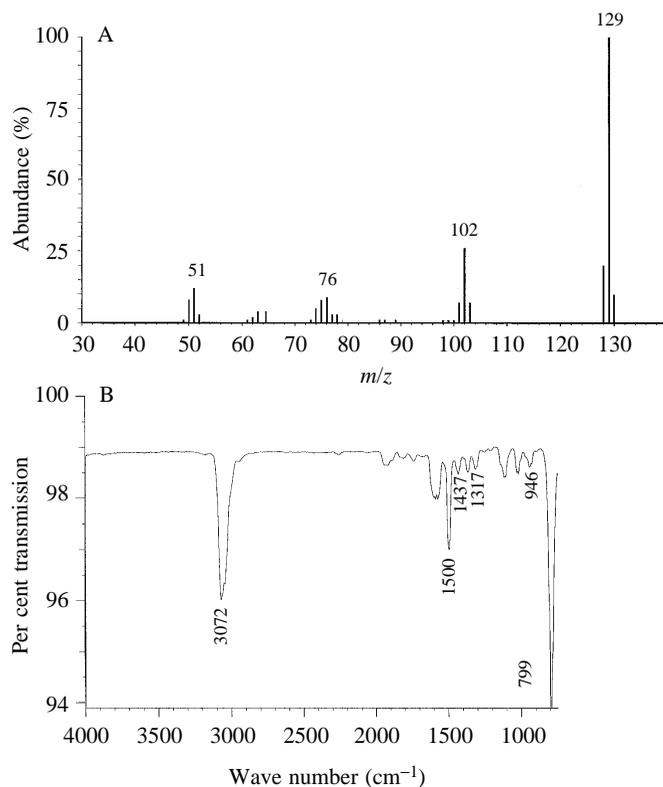

Fig. 2. 70 eV electron-impact mass spectrum (relative intensities normalized to 100 for the most abundant ion) (A) and gas-phase infrared spectrum (B) of the major component in the defensive secretion of *Oreophoetes peruana*. Analytical conditions are given in the text. *m/z* is the mass/charge ratio.
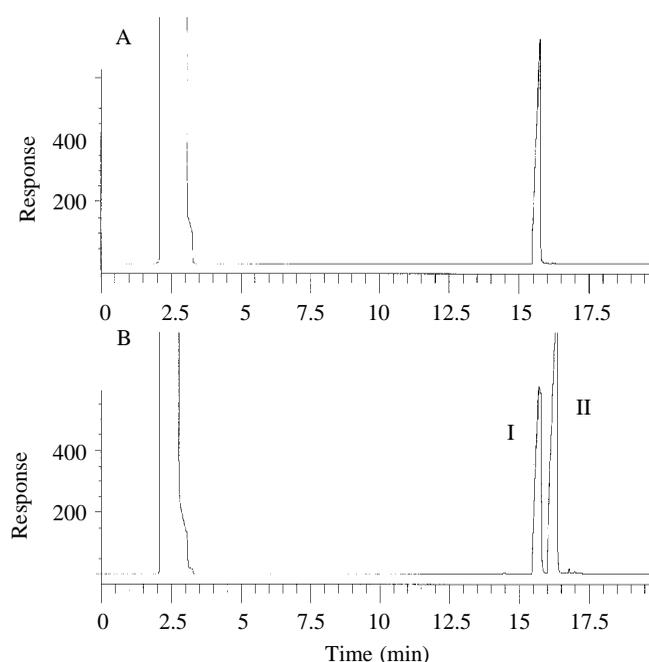

Fig. 3. Gas chromatogram of *Oreophoetes peruana* secretion extract (A) and of a mixture of quinoline (peak I) and isoquinoline (peak II) (B). (Arbitrary units; only relative intensities are significant). The peak at 2.5 min is the solvent (dichloromethane). Analytical conditions are described in the text.
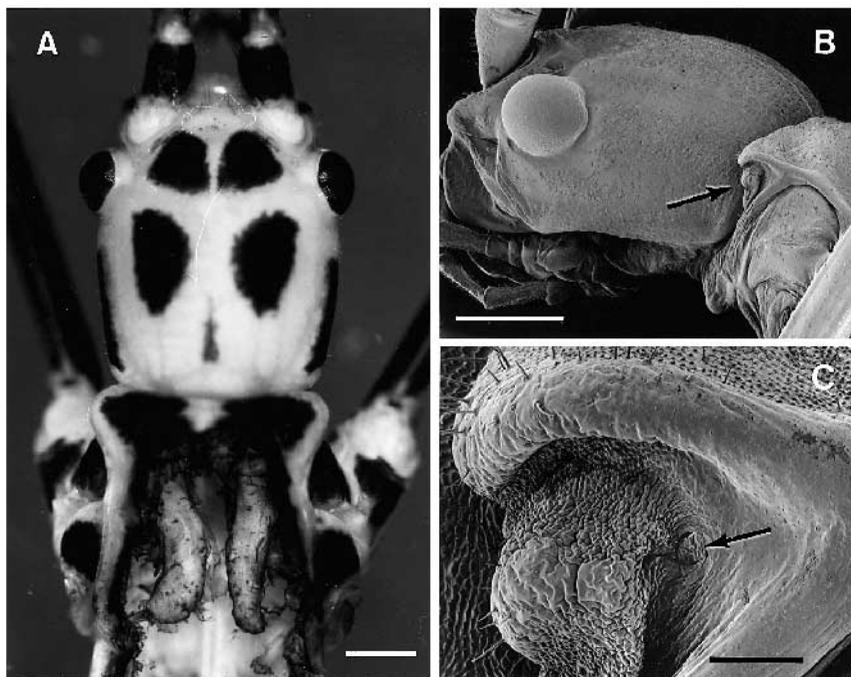
Fig. 4. *Oreophoetes peruana*. (A) The head and prothorax in dorsal view; the pronotum has been dissected away to reveal the two glands. (B) Lateral view of the head and prothorax; the arrow points to the region (enlarged in C) of gland opening. (C) The region of gland opening (arrow). Scale bars, A, B, 1 mm; C, 0.1 mm.

peruana, showed an absence of quinoline, indicating that the phasmid itself probably synthesizes the compound.

The glands

The glands form a pair of identical sacs, lying side by side in the prothorax (Fig. 4A) and opening by way of two minute curved slits just beneath the anterolateral corners of the pronotum (Fig. 4B,C). Each gland consists of a storage sac, invested with compressor muscles, and a slender ejaculatory duct (Figs 5A, 6A). Being integumental organs, the glands are lined in their entirety with cuticle. Treatment of the glands with dilute aqueous potassium hydroxide permitted isolation of this
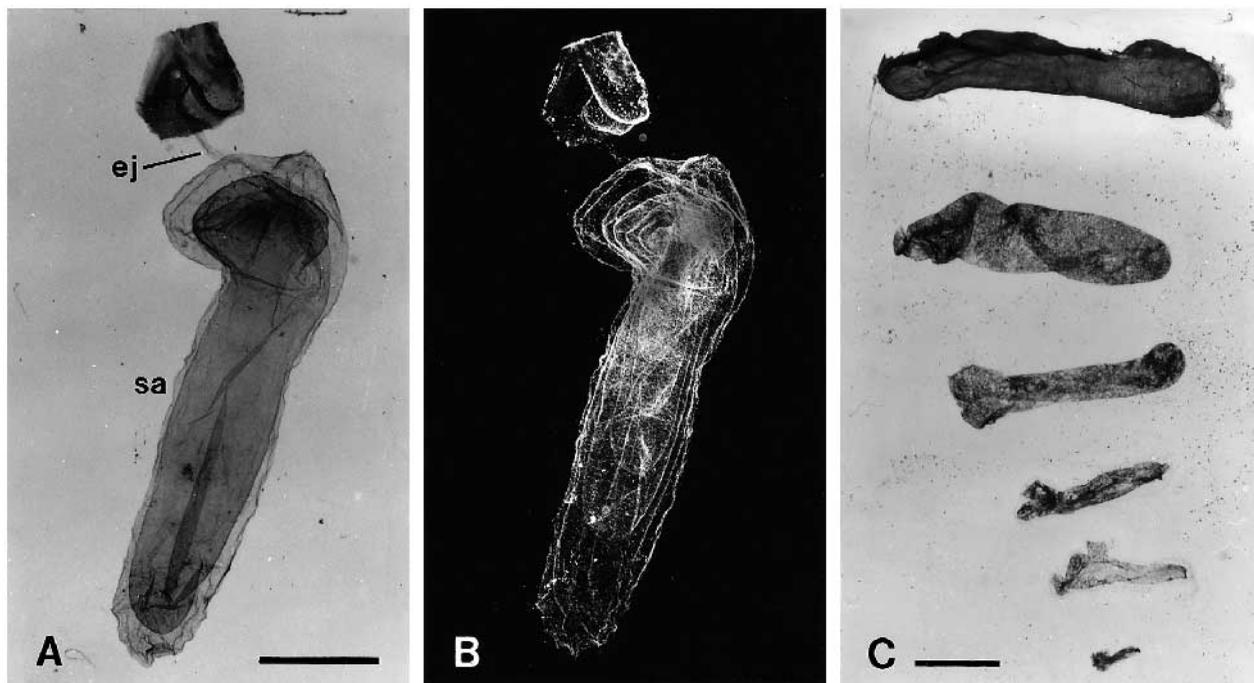

Fig. 5. *Oreophoetes peruana*. (A) Gland, showing storage sac (sa) and ejaculatory duct (ej); the duct leads to a piece of body cuticle on which the gland opening can be seen as a faint white dot (preparation treated with potassium hydroxide and consisting of cuticle only). (B) The same preparation, in dark-field illumination, showing the outlines of shed cuticular linings within the glandular sac. (C) Gland of an adult female (top), with the five cuticular linings dissected from its lumen. Scale bars, A, C, 1 mm.

Fig. 6. *Oreophoetes peruana.* (A) Whole mount of the midportion of a gland, showing the compressor muscles (polarized light). (B) Surface view of the cuticular lining of the gland (preparation treated with potassium hydroxide); note the numerous cuticular ducts, each presumably denoting the site of a secretory cell. Scale bars, A, 0.5 mm; B, 0.05 mm.
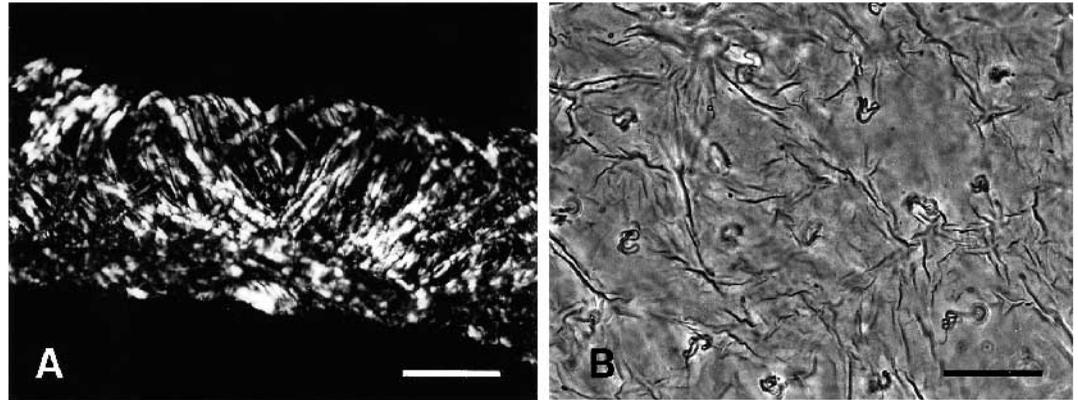
cuticle which, upon microscopic examination proved to be beset with tiny tubules (Fig. 6B), such as are commonly associated with insect exocrine cells (Eisner *et al.* 1964; Happ, 1968; Noirot and Quennedey, 1974). The cellular elements responsible for producing the secretion in *O. peruana* are therefore likely to be part of the epithelial lining of the sacs.

We expected the glands of *O. peruana* to shed their cuticular linings at molting. However, when we examined discarded nymphal skins, we found no vestiges of such linings at their predicted sites within the prothorax. There were only remnants of the linings of the ejaculatory ducts, in the form of extremely fine filaments extending inward from the gland openings. The linings of the sacs themselves, it seemed, had become detached from the linings of the ejaculatory ducts and been left behind within the glands. We postulated that the molting of the glands might proceed in accordance with the scheme depicted in Fig. 7, which predicted that within the glands of any one instar one should find, stacked one within the other, the molted linings of all preceding nymphal stages. This turned out to be true (Fig. 5B). Glands of adult males and females contained as many linings as the number of developmental instars (five) (Fig. 5C). Surface examination of these linings under high magnification revealed vestiges of the cellular tubules, providing added evidence that the linings are indeed shed cuticle.

Bioassays

Quinoline proved effective in all assays (Fig. 8). The 98 of 100 ants that responded to the chemical were all repelled in 2 s or less. Most spiders (*N*=7) extricated their chelicers and moved away from the mealworm within less than 2 s of
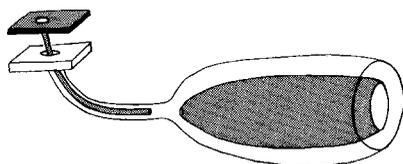
quinoline application; the remainder (*N*=6) took 4–31 s to free themselves, but all had begun attempts to withdraw the chelicers the moment they were stimulated. Most of the spiders dragged their mouthparts in the sand at the bottom of the cage after dropping the prey. With the cockroaches, quinoline was effective on contact and near-contact, as was the *O. peruana* secretion itself. All five frogs scratched the site of quinoline application, using the ipsilateral hindleg (four hopped away after scratching; one hopped away and then scratched). The control (water) was ineffective or virtually ineffective in all four assays. Neither the spiders nor the frogs showed delayed ill-effects from the tests. All were released at their sites of capture after several days.

Discussion

O. peruana is unusual on two counts: the molting mechanism of its glands, and the chemistry of its secretion.

By not shedding the cuticular lining of its glands, *O. peruana* avoids losing its secretory supply at molting. Were the linings to be drawn from the body as intact pouches, their contents would be shed as well, and the animal would be left chemically defenseless following the molt, until it reloaded its glands. The
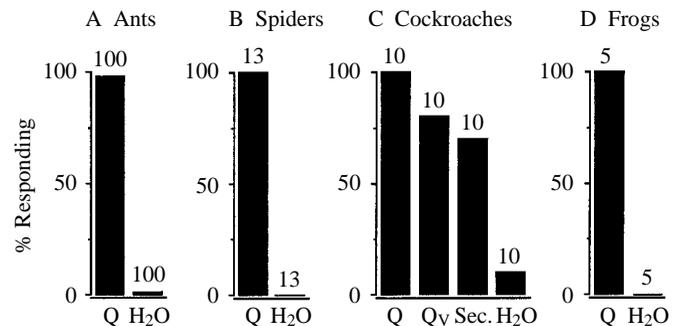

Fig. 7. Diagram of the *Oreophoetes peruana* gland, in the process of molting. The cuticle being shed is denoted in gray.

Fig. 8. Deterrency of quinoline to four test animals. The chemical was applied directly as a liquid (Q), or on near-contact as a vapor (Q$_v$), or as part of a sample of *Oreophoetes peruana* secretion (Sec.). Water served as a control. Numbers above bars indicate sample size. Details are given in the text. In all assays, *P*<0.001; tests of independence (*G* statistic with Williams' correction).
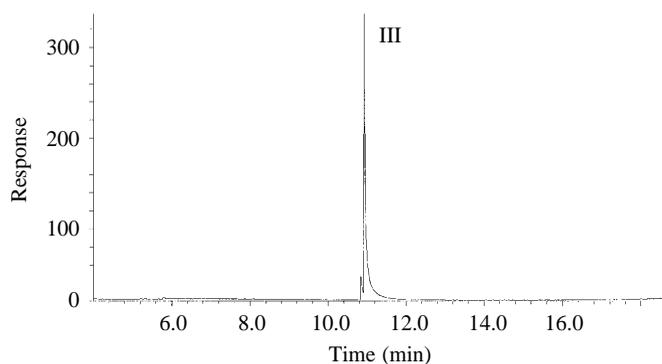
Fig. 9. Reconstructed gas chromatogram (GC-MS analysis) of an extract of a sample of secretion from *Anisomorpha buprestoides* (DB-5-coated fused-silica column; oven temperature was kept at 60 °C for 1 min, then raised by 15 °C min^{-1} to 270 °C). (Arbitrary units; only relative intensities are significant). The peak labeled III is anisomorphal.

linings of the sacs are probably too bulky to be drawn through the narrow ejaculatory ducts at molting. We envision that they simply tear away from the duct linings when these are pulled from the body. The severance leaves the cuticular pouches within the glands perforated, ensuring that their contents mix with secretion newly produced after the molt.

The molting mechanism we postulate for *O. peruana* predicts that the animals should be unable to discharge during the molt, when the ejaculatory ducts could still be plugged by their shed linings, but able to discharge following the molt, when these linings have been extricated. Both these predictions were confirmed.

Other insects that possess defensive glands in the nymphal stage may lack provision for salvaging their secretion when they molt. In the cockroach *Diploptera punctata*, for instance, the lining of the two quinone-discharging glands are shed, together with their contained secretion, at each molt (Roth and Stay, 1958; Baldwin *et al.* 1990). It takes the cockroaches 8–24 h to reload their glands, during which time they are vulnerable to predation (Eisner, 1958).

Given the proven potency of quinoline in our bioassays, the compound must serve *O. peruana* well. Against ants, quinoline proved effective on near contact as a vapor. Ants could therefore be repelled not only by the discharges themselves, but by residual secretion remaining on the phasmid's body following ejections. Given that ants may attack in groups, this could be of particular importance. In the cockroach test, quinoline was active both on contact and near-contact. While
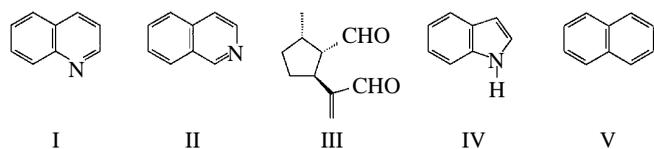
cockroaches are obviously not themselves enemies of phasmids, their topical sensitivity to quinoline could be indicative of a general insect sensitivity to the compound. Indeed, for a number of defensive substances of arthropod origin, including quinones and isoprenoids, potency in the cockroach scratch test has been shown to correlate with insect repellency (Smolanoff *et al.* 1975; Eisner *et al.* 1986, 1996; Peschke and Eisner, 1987). Quinoline in the actual secretion of *O. peruana* does not occur in pure form. However, it is not strictly dissolved in the fluid but is present as the inner phase of an emulsion. In actuality, therefore, the quinoline in discharged secretion can be envisioned to act at full strength, albeit discontinuously over the surface of contact of the fluid. Notably, in the test with cockroaches, the actual secretion proved almost as potent as pure quinoline. Also of importance is that the outer phase of the secretion (presumably water) is more volatile than quinoline. Within droplets of discharged secretion, therefore, when these contact a target, quinoline can be expected to undergo a rise in concentration. In small droplets, such a rise could occur rapidly. Being an aqueous emulsion could also help the secretion spread over wet surfaces, including not only amphibian skin, but also the oral linings and exposed ocular surfaces of birds and mammals. While we would have liked to test *O. peruana* with actual predators, we had insufficient phasmids to do so.

Quinoline derivatives, presumed to serve for defense, have been isolated as systemic or glandular factors from a number of insects. Thus, for instance, *N*-α-quinaldyl-L-arginine–HCl, methyl-8-hydroxyquinoline-2-carboxylate and 1-methyl-2-quinolone have been reported, respectively, from a coccinellid beetle *Subcoccinella 24-punctata* (Wang *et al.* 1996), a dytiscid beetle *Ilybius fenestratus* (Schildknecht *et al.* 1969) and a lycid beetle *Metriorrhynchus rhipidius* (Moore and Brown, 1981). Many other quinoline derivatives are known from plants and microorganisms (Buckingham, 1994).

Quinoline itself is a rare natural product. The compound was discovered by Runge in 1834 as a minor constituent of coal tar (Jones, 1977). It was first isolated from a plant source almost a century later, when Späth and Pikl (1930) found it to be a trace constituent (≈0.003 %) of angostura bark (*Galipea officinalis*). *O. peruana* appears to be the first known animal source of this simple heterocycle.

Other phasmids that possess defensive glands secrete substances unrelated to quinoline. Compounds characterized so far from phasmids are all cyclopentanoid monoterpenes (e.g. anisomorphal, nepetalactone), including monoterpene alkaloids (e.g. actinidine) (Meinwald *et al.* 1962; Eisner, 1965; Smith *et al.* 1979; Chow and Lin, 1986; Ho and Chow, 1993). Not all these phasmids share the gland-molting mechanism of *O. peruana*. *Anisomorpha buprestoides*, for instance, the source of anisomorphal, has thoracic glands essentially like those of *O. peruana*. However, its ejaculatory ducts are relatively wide, and its glandular sacs contain no traces of shed cuticular linings, indicating that these linings are probably drawn whole from the glands at molting (T. Eisner, unpublished data).

Fig. 10. Chemical formulae for quinoline (I), isoquinoline (II), anisomorphal (III), indole (IV) and naphthalene (V).

A point of interest concerning the *O. peruana* secretion, quite aside from the nature of its active constituent, is that this chemical should be present in the fluid with virtually no accompanying components. Insect secretions, whether defensive or pheromonal, are usually mixtures, often of some complexity (Bettini, 1978; Blum, 1981; Mayer and McLaughlin, 1991). Phasmids themselves may produce multiple-component defensive secretions (Smith *et al.* 1979; Ho and Chow, 1993). However, there is at least one phasmid that is also exceptional: *Anisomorpha buprestoides*. We recently re-examined a sample of secretion from this insect and found the fluid to contain only slight traces of volatiles beside anisomorphal (Fig. 9).

It is interesting to compare quinoline with two closely related compounds, indole and napththere (Fig. 10). Indole is well known from bacterial, plant, and animal sources (Buckingham, 1994). It is a trace constituent of the pygidial gland secretion of several ant species (Billen *et al.* 1988; Jackson *et al.* 1990) and of a caddisfly *Pycnopsyche scabripennis* defensive secretion (Duffield *et al.* 1977). Indole has also been reported recently (without functional assignment) to be present in 'huge quantities' in a scarab beetle *Holotrichia consanguinea* (Leal, 1997). From a structural and electronic viewpoint, the closest chemical relative of quinoline may be naphthalene, the single most abundant chemical ($\approx 11\,\%$) in coal tar. Michael Faraday determined the composition of naphthalene in 1826 (Cuffey, 1977), and it subsequently gained great popularity as a household insect repellent.

Whereas indole appears to owe its biosynthetic origin to the action of a tryptophanase on the amino acid tryptophan (Leal, 1997), quinoline has no comparable natural amino acid precursor and must be produced by *O. peruana* in some other way. Anthranilic acid, which serves as the precursor of arthropod-produced quinazolines (Schildknecht and Wenneis, 1967), is a likely starting material. The monoterpenes produced by other phasmids appear to be synthesized from mevalonic acid by the conventional terpenoid biosynthetic route (Meinwald *et al.* 1966). By diverging from this scheme and secreting quinoline, *O. peruana* appears to have hit upon the expedient of producing an insect analogue of 'moth balls'.

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