Chemical Composition and Behavioral Responses of the Marine Insect *Halobates hawaiiensis* (Heteroptera: Gerridae)

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Halobates is the only insect genus with representatives in the open ocean. How adults find one another at sea has long been an intriguing issue. Since chemical communications have been demonstrated in a related marine veliid *Trochopus*, and laboratory bioassays indicated behavioral differences between males and females when insect extracts were presented, we carried out similar studies on *Halobates*. Analyses of surface lipid constituents of female and male *Halobates hawaiiensis* revealed marked differences. Palmitic and oleic acid, major constituents in the male extracts, were absent in the female extract, whereas nonacosenol, dominating the female extracts, was not detected in the male extracts. Analyses of nymphal extracts indicated an intermediate chemical profile. Surface waxes of all insect stages investigated showed nonacosanol and isononacosanol to be main components. "Headspace" analyses of airborne chemicals showed high levels of 4-hydroxy-4-methyl-2-pentanone and benzaldehyde from the male, whereas benzyl alcohol was the main component in the female mixture.

Introduction

The genus *Halobates* (Heteroptera: Gerridae), includes over 45 species, 5 of which are pelagic, and each has a distinct, well-defined distribution pattern. They are rather small insects; adults measure 5–6 mm long. They are totally wingless during all stages of their life cycle and live entirely at the sea-surface, feeding either on terrestrial insects or on zooplankton trapped at the sea-air interface (Cheng, 1973a; Andersen and Polhemus, 1976). They are in turn preyed upon by seabirds and surface-feeding fishes (Cheng, 1985). Like other gerrids, sea-skaters are hemimetabolous, with a life cycle consisting of the egg, 5 nymphal instars and the adult. The total developmental period from egg to adult may last from 35 to 70 days, depending on seawater temperature (Cheng, 1981; Cheng and Frank, 1993). Adults are sexually dimorphic, the female being usually larger (Spence and Andersen, 1994). Males have specifically modified external genital features which we can use for specific identification (Herring, 1961). Some of their special adaptations to an oceanic existence include egg-laying on floating substrates (Cheng, 1973a), an air-trapping velvety layer surrounding the body to prevent drowning (Cheng, 1973b), storage of triglycerides which enables them to withstand periods of food shortage (Lee and Cheng, 1974), and a highly UV-absorbing layer in the cuticle to shield them against radiation damage (Cheng *et al.*, 1978).

Most species of *Halobates* occur near shore, often associated with coastal mangroves. The five open-ocean species occupy large areas of the Atlantic, Pacific and the Indian Oceans, extending from 40 °N to 40 °S (Cheng, 1989). Males and females have to find one another in order to mate. This ability is probably assisted by a highly developed chemoreception that allows the detection and recognition of conspecifics over long distances (Harder *et al.*, 1996). Since at the sea surface an airborne attractant might be too readily dispersed by wind and thus unnecessarily wasteful to synthesize, we can postulate that a surface-dispersible agent would be preferable, presumably operating most effectively on still water during periods when the sea is calm.

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One of the most intriguing problems concerning *Halobates* behavior is how the sexes meet amid storms and waves on the open-sea (Cheng, 1985). Whereas in some freshwater gerrids surface ripples generated by the males serve for mate attraction (Wilcox, 1972), this system is unlikely to work in an oceanic environment with turbulent currents and waves. Circumstantial evidence from field studies on the physiological ecology of *Halobates robustus*, carried out in the Galàpagos Islands indicated that there may be chemical communications between individuals (Birch *et al.*, 1979).

Sex pheromones are probably the most widely studied of insect semiochemicals (Aldrich, 1988). Most are airborne, produced by the female, and some may be detectable by males several kilometers downwind from the source (Murlis *et al.*, 1992). On the other hand, male-produced pheromones are rare, and not all are sex-specific; some may act to promote aggregation rather than attracting females (Landolt, 1997). The majority of identified insect sex pheromones comprise long-chain unsaturated alcohols, acetates, aldehydes and carboxylic acids. Each species of insect may blend such compounds in various proportions to produce a specific sex pheromone (Roelofs, 1995).

Although sex attractants have been demonstrated in a number of marine invertebrates (Alcock, 1994), only a few have been identified chemically. During the course of our investigations on insect communication systems and isolation of bioactive natural products (Agalias *et al.*, 2000; Ortiz *et al.*, 2000) we studied the small marine veliid *Trochopus plumbeus,* a near-shore relative of *Halobates*, and found clear evidence for a chemical agent whereby males attracted females (Cheng and Roussis, 1998). In the present study we report on similar investigations and field behavioral experiments carried out on *H. hawaiiensis.* Although we observed responses that could be attributed to chemical reception among individuals, and analyses of volatile metabolites that could be extracted from surface lipids of the insects revealed marked chemical differences between males, females and nymphs, interactions between opposite sexes were found to be more complex.

Materials and Methods

Since we could not carry out behavioral studies at sea, we chose to study the coastal species, *H. hawaiiensis*, which occurs around the coasts of the Hawaiian Islands and French Polynesia. Bioassays and field experiments were carried out in May, 1999 in Moorea, Tahiti, where laboratory facilities were available at the Gump Biological Station operated by the University of California, Berkeley. The Gump station is situated on the west bank of Cook's Bay, on the north side of the island, in a small inlet surrounded by sparse coastal mangrove and with open access to the main bay. To attract the insects we used three 1-kw halogen lamps hang under a wooden pier at the station, about 1-m above the sea surface. Collections were made nightly for 2–3 h . The lights were switched on 30 min before sunset. Insects were captured by plastic scoops (20 cm diameter, 1-mm mesh) or with small aquarium fish nets, and separated into adult males, females and nymphs. Groups of 20 or more of each category were kept in separate aquaria half-filled with clean seawater. They were allowed to acclimatize in captivity, and were fed daily with live flightless or frozen fruit-flies until used for bioassay experiments.

Chemotropism experiments

Extracts of *Halobates* in a 1:1(v/v) methanol: dichloromethane mixture were applied to filter-paper strips (2 cm × 5 cm). The solvent was allowed to evaporate and the strips were then used for the experiments employing either one or two beakers.

In the single-beaker system the test-strip and insect were introduced simultaneously at opposite sides of the same beaker. A positive reaction was indicated by repeated contact of the insect with the filter paper. For the double-beaker system we used two polypropylene beakers (2-litre, Nalgene) connected by a horizontal channel of plastic (PVC) tubing, 12 cm long, 2.5 cm diameter. Fresh seawater was poured into the beakers until the water level half-filled the connecting tubing, forming a channel where a flat, undisturbed water surface was maintained. For each assay, insects of one sex were introduced into one beaker and a test strip into the other, simultaneously. Movements of insects were observed, and numbers in each beaker were recorded every 30 seconds for a total of 2 minutes. A single adult was placed in one beaker and filter paper impregnated with different con-

centrations of extracts of live insect extracts, of the same or opposite sex, in the other. Each experiment was repeated 10 times, different insects being used each time.

Chemical studies

20 live insects were used (males, females or nymphs) for each of the extracts listed below:

1. Extracts were prepared by immersing insects for 2 h in a mixture of 1:1(v/v) methanol: dichloromethane, removing the insects and evaporating most of the solvent at atmospheric pressure, using a 'vigreux' column to retain volatile metabolites.
2. For surface waxes, insects (males, females or nymphs) were rinsed for only 5 sec in hexane, and the solutions were concentrated as described above.
3. For "headspace" analysis, insects (males or females) were placed in an air-tight closed-loop "stripper", and the air of the system was continuously passed through a glass trap loaded with 1.0 g of activated Tenax TA 70 polymer. After one hour the glass traps were removed, washed with small volumes of hexane. The extracts were stored at −4°.

All subsequent chemical analyses were carried out in the Laboratory of Pharmacognosy, University of Athens, Greece. Gas chromotographic (GC) analyses were performed on a SRI 8610C GC equipped with a split/splitless injector (250 °C) and a flame ionization detector (280 °C). We used He (2 mL/min) as carrier gas with DB−5 (30 m X 0.32 mm) and HP-Innowax (30 m X 0.25 mm) capillary columns. The initial temperature of the columns was 60 °C, and they were then slowly heated at the rate of 3 °C/min to 280 °C. Mass spectra were obtained by using a Hewlett Packard 5973−6890 GC-MS system equipped with a HP 5MS 30 m X 0.2 μm thick film operating on EI mode. The capillary columns used and the temperature program were similar to those for the GC. Identifications of chemical constituents were based on comparisons of the Rt values and mass spectra with those obtained from authentic samples (Adams, 1995) and/or NIST/NBS and Wiley library spectra.

Results and Discussion

Field observations

The only locations where *Halobates* were observed during the day were at Cook's Bay (adults), Temae Beach (adults) and around mangroves (nymphs) at Haapiti Village on the south-western shore of the island. At the Gump station no insects were seen when the lights were turned on shortly after sunset; the first insects arrived approximately 10 min later. About 75% of the insects caught were adults (58% males); the rest were nymphs of all developmental stages. There may be no marked reproductive seasonality for this species in Moorea.

Biological assays

A variety of bioassays were carried out using combinations of insects (males, females, nymphs) and extracts of varying concentrations equivalent to 0.1 to 2.0 individuals per test strip. Adults were found to be extremely sensitive to light. We noted that the insects would move towards whichever beaker was exposed to a brighter light. When one of the beakers was covered and thus darkened, the insects would move to the exposed beaker within 1−2 seconds. Accordingly, for all subsequent experiments we made sure that the beakers were evenly lighted.

Experiments performed using a single beaker or the two-beaker systems produced similar results (Table I) and confirmed the ability of *H. hawaiiensis* to detect chemical signals and express different behavioral responses. Although the assays also showed some evidence for repulsion as well as attraction between adults, they were not consistent, possibly being influenced by the physiological state of the adults (e.g. newly molted and not sexually mature, sexually mature, gravid). However, though we dissected many females, we found no correlation between behavior and their physiological state.

Table I. Behavioral responses.

Insect	Extract	Reaction	Concentration
Female	male	attraction	≥ 0.2
Female	female	repulsion	0.2
Male	male	repulsion	≥ 2.0
Male	female	attraction	≥ 2.0

Table II. Percentage composition of whole insect extracts.

Constituent	Ret. Time (min)	Female	Male	Nymphs
Glycerol	7.09		tr	
2-Cycloheptenone	17.92		tr	
2,7,10-trimethyl Dodecane	34.79		0.4	
Tetradecanoic acid	36.92		1.17	
Adenine	38.27		0.14	
Hexadecene	41.29			3.22
Hexadecanoic acid methyl ester	42.58		0.2	
Hexadecanol	43.18		2.33	
Palmitic acid	43.95		10.8	2.55
n-Heptadecanol	47.07		tr	4.82
Octadecenol	47.46			1.87
Octadecanol	48.03			1.57
(Z-Z)9,12 Octadecadienoic acid	49.35		3.0	0.88
Oleic acid	49.41	tr	10.45	0.77
Hexadecanedioic acid methyl ester	57.05		tr	
Octadecane	58.28	0.40		
Eicosane	65.68			0.49
Z,Z,Z-11,14,17 Eicosatrienoic acid methyl ester	68.11	0.7	2.6	2.03
Nonacosenol	68.74	12.9		13.5
Nonacosanol	69.02	16.2	16.94	7.53
Isononacosanol	69.22	23.26	15.87	18.91
Tricontane	70.40	12.13	8.05	7.67
Dotriacontane	71.39		1.3	
Cholest-5-en-3-ol	74.48	9.96	6.6	8.85
Tetratriacontane	74.71	1.01	4.6	1.3
Total		76.16	84.45	75.96

Chemical analyses

A total of 25 components were detected and quantified in the investigated extracts (Table II). The majority of the identified compounds comprised linear-chain alcohols, acids and acid esters. Most of the identified compounds are relatively insoluble in water, but they bear a polar functional group that may promote their dispersion over a water film, so that they could act as surface 'messengers'. We found significant qualitative and quantitative differences between the male and the female extracts. Nymphal extracts had similarities with both male and female extracts.

The male extract showed the most complex chemical profile. A major difference between the sexes was the absence of palmitic (hexadecanoic) acid from the female, whereas it was one of the major constituents of the male extract (10.8%). (Palmitic acid has previously been identified as a component of the male sex pheromone of the dung beetle *Khefer lamarcki* by Mayer and McLaughlin, 1990). The male extract also contained tetradecanoic acid (1.17%) and oleic acid (10.45%) which were detected only as traces in the female extract (Table II). Nonacosanol and isononacosenol were significant components of all analyzed extracts, whereas nonacosenol, dominating the female extract (12.9%) was not detected in the male extract.

The surface waxes of all investigated specimens were, as expected, rich in hydrocarbons, long-chain lipid acids (C20) and alcohols such as nonacosanol and isononacosanol (Table III). The female surface wax contained much nonacosenol, octadecane and nonadecene. A striking difference between male and female extracts was the presence of a high proportion of tricontane in the former and nonacosenol in the latter.

The headspace analyses (Table IV) showed 4-hydroxy-4-methyl-2-pentanone (51.66%) to dominate male airborne emissions; whereas in the female profiles benzyl alcohol (10.26%), butyl carbitol (18.05%) and tridecane (15.85%) were the most significant contributors.

Table III. Percentage composition of surface waxes.

Constituent	Ret. Time (min)	Female	Male	Nymphs
Cyclohexane	3.21	4.30		
Heptanal	4.25	0.17	tr	1.00
Octanal	6.8	tr	tr	
Heptanoic acid	9.30	tr	tr	0.30
Nonanal	10.26		tr	0.60
Decanal	14.39			tr.
Nonanoic acid	17.28		tr	
E-4-Undecanal	18.08			0.37
n,n diethyl Toluamide	29.9		tr	
Dodecanol	31.2		tr	
Isopropyl myristate	39.1		tr	
Tetradecanal	45.64			0.8
Hexadecane	48.2		tr	
Pentadecanal	51.97	0.31	0.3	1.6
Hexadecanal	57.78	1.3	0.64	3.3
Octadecane	58.28	10.04	1.6	
Nonadecene	64.5	7.54	1.4	1.94
Nonadecane	65.40			0.9
Eicosane	65.68	0.9	1.2	0.11
Eicosanol	66.74	0.8	0.71	
Z,Z,Z-11,14,17 Eicosatrienoic acid	67.94	1.3	2.26	
Z,Z,Z-11,14,17 Eicosatrienoic acid methyl ester	68.11	1.16	3.7	1.16
Nonacosenol	68.74	16.43		14.76
Nonacosanol	69.02	14.16	19.32	14.83
Isononacosanol	69.22	13.21	19.50	29.3
Tricontane	70.40	1.64	20.70	16.62
Tetratriacontane	74.71	3.1	6.42	1.5

Table IV. Percentage composition of headspace gases.

Constituent	Ret. Time (min)	Female	Male
4-Hydroxy-4-methyl-2-pentanone	3.30		51.66
Benzaldehyde	5.65	2.01	4.61
Hexanoic acid	6.04	5.78	
Phenol	6.18	0.68	
Benzyl alcohol	7.81	10.26	2.38
Nonanal	10.29	4.33	1.4
butyl-Carbitol	13.70	18.05	
Tridecane	18.39	15.85	4.63

The results obtained from this study indicate that there could be chemical communications between adult males and females of *Halobates hawaiiensi*. We found evidence for repulsion as well as attraction between the sexes. Major lipid constituents in the male extracts could be readily dispersed over a surface film, and thus presumably could operate more effectively on calm seas than airborne semiochemicals. Further studies using electro-antennogram and bioassays with chromatographic fractions of the extracts will have to be conducted in the field before the chemical nature and activity of any pheromone(s) could be fully defined.

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