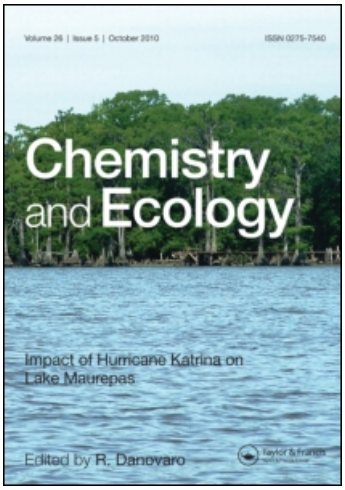

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Naik, D. G. , Babrekar, A. A. and Nath, B. B.(2006) "Pheromone-like' compounds in the cuticle of aquatic *Chironomus* larva', Chemistry and Ecology, 22: 6, 501 — 508
To link to this Article: DOI: 10.1080/02757540601025014
URL: http://dx.doi.org/10.1080/02757540601025014

Chemistry and Ecology
Vol. 22, No. 6, December 2006, 501–508

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'Pheromone-like' compounds in the cuticle of aquatic *Chironomus* larva

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(Received 31 May 2006; in final form 28 August 2006)

Chironomid midges are the most commonly found dipteran insects in all types of aquatic ecosystems. Cuticular extract was bio-assayed, and it exhibited enhanced attraction to the larvae. Therefore, it was subsequently analysed by gas chromatography–mass spectrometry. Two compounds were identified as farnesol and farnesene. Bioassay of farnesol indicated its attractive properties. The components present in the cuticular extract can, therefore, be considered as pheromone-like compounds.

Keywords: *Chironomus*; Pheromone; Cuticular extract; GC-MS; Farnesol

1. Introduction

Insect pheromones coordinate a large number of biological activities. These chemical signals are emitted for various purposes, and accordingly the pheromones are classified as aggregation pheromone, alarm pheromone, territorial pheromone, trail pheromone, sex pheromone, and egg-laying-deterrent pheromone [1].

Insect pheromones are airborne signal molecules. There have been no reports of any insect pheromones acting in the aquatic environment. However, among other arthropods, the crustaceans are known to have water-borne pheromones [2]. In the lobster, *Homarus americanus*, mate location has been shown to be mediated through chemical cues [3]. Female-derived sex pheromones have been reported from the shore crab *Carcinus maenas* [4]. In another crustacean, although the sex pheromones have been found to be released by the female crayfish *Pacifastacus leniusculus*, its chemical identification has not yet been established [5].

The current study deals with one of the most ubiquitous groups of dipteran insects, *Chironomus*, commonly known as 'non-biting midge'. These insects are abundantly found in all types of freshwater ecosystem and can also thrive under extreme environmental conditions [6]. Aquatic larval stages of *Chironomus* constitute a major part of their life cycle. This unique group of insects has never been explored for the presence of pheromones. The present study

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Chemistry and Ecology
ISSN 0275-7540 print/ISSN 1029-0370 online © 2006 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/02757540601025014

was motivated by a previous report, where the aquatic larvae of *Chironomus* were shown to recognize their self-built housing tubes [7]. As for the other terrestrial insects, cuticular pheromones have been implicated for homing as well as aggregation behaviour [1, 8]. Using a similar line of argument, the role of cuticular pheromone in the 'tube recognition' behaviour of the aquatic larvae of *Chironomus* can be speculated. As a preliminary step towards understanding the chemical basis of larval behaviour in *Chironomus*, we have examined the extracts of the larval cuticle, along with the housing tubes, with an aim to explore their 'pheromone-like' properties.

2. Materials and methods

2.1 *Chironomus rearing*

A laboratory-reared inbred line of *Chironomus ramosus* was used which originated from a natural population of tropical midge [9]. This line has been routinely maintained for the last 10 yr under laboratory conditions. Rearing was carried out by following the method described by Nath and Godbole [10]. Larval and pupal stages are aquatic, whereas adults are terrestrial. The life cycle of *Chironomus ramosus* from egg to imago is completed in 35 d. Larvae and pupae were reared in enamel trays, whereas the adults were maintained in cages for the continuation of the life cycle. The rearing trays and cages were maintained in a temperature-controlled room ($26 \pm 1\,^{\circ}C$) with 12 h dark and 12 h light phases. For the maintenance of the culture, freshwater was replenished every day, and a fine paste of moss was added to these trays.

2.2 *Sample preparation*

Fourth instar larvae from a synchronous culture were picked up and kept separately in a bowl. Washed and dried sand particles (diameter less than 1 mm) were provided to them. Larvae constructed compact tubes within 3 h and became acclimatized. Such larvae ($n = 50$) were taken out from the tubes and were dissected in insect ringer solution. The visceral material was removed by applying gentle pressure from the tail end to the proximal end. The cuticle with an intact tail and mouth end was removed and dropped into cold acetone ($500\,\mu l$). In order to avoid any possible loss of volatile compound during processing, the extract was centrifuged in cooling centrifuge (Kubota 3700) at 1000 rpm for 5 min, and the supernatant was used directly for the first bioassay and the gas chromatography-mass spectrometry (GC-MS) analysis. Preparation of three sets of samples was carried out.

2.3 *Crude-extract bioassay*

The design of the bioassay was based on the innate and obligatory larval behaviour of the tube-building habit. Larvae cannot survive without making a tube in which to live at any stage of the development. Initially, a bioassay of the extract of cuticle was carried out. For the bioassay, a glass Petri dish (11.5 cm diameter) was used. Cuticular acetone extract ($20\,\mu l$) was coated on one half of the Petri dish. The other half was not coated with anything. The setup was left undisturbed for 2–3 min to dry. The Petri dish was then filled with water (80 ml) containing a uniform layer of the sand particle (7 g) as tube-building material. Fourth-instar larvae ($n = 10$) were released at the centre of the dish, located at the centre of the Petri dish (figure 1a). They were observed after 3 h, when they settled down building individual tubes at the chosen zone. In this experiment, the number of tubes built by the larvae were the end-point

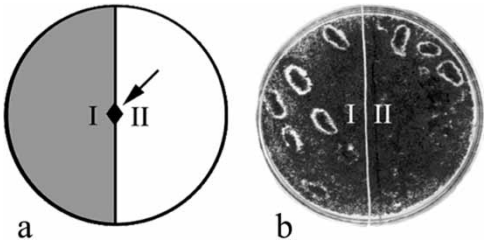

Figure 1. Bioassay of crude cuticular extract on *Chironomus* larvae. (a) Schematic design of the bioassay showing half of the glass Petri dish (I) coated with the crude extract and (II) the other half left uncoated. The arrow indicates the 'centre of the dish', from where the larvae were released for the bioassay. (b) Photograph showing the end-points of the bioassay.

of the assay as an indicator of larval preference towards coated and uncoated zone. The tubes made in the coated and uncoated zones were counted. A blank experiment was also carried out as a reference, in which only acetone (20 μl) was coated on one half of the Petri dish. The bioassays were conducted during the same time of day in the laboratory. Both uncoated and coated zones were equally illuminated during the bioassay. This ensured that environmental parameters like light did not play any role in influencing larval choice for a particular zone in the assay plate. A uniform temperature of 26 ± 1 °C was maintained during the experiment. Five replicates of each of the bioassay were carried out.

2.4 *Gas chromatography–mass spectrometry*

Three fresh samples of the cold cuticular extract (2 μl) were directly analysed by GC-MS. The GC-MS analysis was carried out on a Shimadzu QP-5000 spectrometer using a capillary column DB-1, having 0.25 mm ID, 30 m length and 30μm film thickness. Mass spectra were recorded at 70 eV. The NIST library was installed for reference. Quantification was not carried out.

2.4.1 GC conditions. Injector SPL-17 in a split mode: split ratio 50, 200 °C, total flow 79.5 ml min^{-1}; column temperature programme: 80 °C (5 min)/80–100 °C (at the rate of 5 °C min^{-1})/100 °C (4 min)/100–150 °C (at the rate of 5 °C min^{-1})/150 °C (10 min)/150–200 °C (at the rate of 5 °C min^{-1})/200 °C (50 min).

2.4.2 MS conditions. Recorded at 70 eV. Mass range 40–450, scan time 1 s, solvent cut 3 min, start time 4 min.

2.5 *Farnesol bioassay*

Farnesol was procured from Aldrich Chemical Company (Milwaukee, WI; cat. no. 27759-1), since one of the constituents of the cuticular extract was farnesol. Bioassays of the various formulations of synthetic farnesol were also carried out in exactly the same way. The varying quantity of farnesol being coated on one half of the petriplate was 0, 4.8, 9.6, 19.2, and 38.5 nl cm^{-2}. The difference (Δ), in the number of larvae getting attracted to coated zone and uncoated zone, indicated the extent of attraction due to farnesol. Five replicates of each bioassay were recorded. The mean number of larvae attracted to the coated and uncoated zones was determined for each concentration. The difference then was treated as attractiveness (Δ).

2.6 *Statistical analysis*

SPSS version 11.0 in Windows platform was used to carry out a statistical analysis of the data. For testing the difference between larvae attracted to the coated and uncoated zones, Wilcoxon's matched-pair signed rank test was used. The data hold true for normality assumption, and Pearson's correlation coefficient was obtained for analyzing the response of larvae at different concentrations. The difference in the number of larvae attracted to coated zone *vis-à-vis* the uncoated zone for different concentrations was tested using Tukey's multiple comparison tests.

3. Results and discussion

In this study, a chemical analysis of the extracts of larval cuticle and the tube (where they live) was carried out in order to evaluate 'pheromone-like' properties. A novel bioassay has been designed to assess the efficacy of the extract (figure 1). In a preliminary bioassay, an almost equal distribution of the larvae (5 ± 0.8) was observed in the blank experiment. Subsequently in the bioassay of cuticular extract, the zone coated with the crude extract was found to contain significantly more larvae (7 ± 0.2) than the uncoated zone (3 ± 0.2 Wilcoxon's matched- pair signed rank test, $N = 5$, $p < 0.05$). Thus, the bioassay of the cuticular extract significantly indicated its pheromone-like attractive property. Having been encouraged by these findings, a GC-MS analysis of the cuticular extract was carried out. Gas chromatograms of three freshly collected extracts were identical and revealed the presence of seven closely related compounds (figure 2). Two of them were dominant (table 1). The fragmentation pattern of these two dominant signals at RT = 43.28 min and RT = 44.34 min were analysed through the NIST library and happened to be 3,7,11, trimethyl-2, 6,10-dodecatrien-1-ol (farnesol) and farnesene. Such long-chain aliphatic compounds are sparingly soluble in water [11]. However, their exact mode of action needs to be investigated. Pheromonal activity of such aliphatic compounds has been demonstrated only from the terrestrial group of insects [12] but not reported from any aquatic stages of insects. In order to confirm the pheromonal property of one of the identified compounds from the cuticular extract, we carried out the farnesol bioassay, the mixture of isomers of the expected compound.

In the Farnesol bioassay, it was observed that a significant number of larvae migrated to the area coated with farnesol in all the replicates. The plot of Δ, attractiveness, against the quantity of farnesol applied to the test zone (figure 3) showed that the attractiveness of farnesol initially increased with the quantity up to 19.25 nl cm^{-2} and thereafter remained almost constant. The correlation between the farnesol concentration and the number of larvae attracted was positive and significant (Pearson's correlation coefficient; $r = 0.826$, $p = 0.001$, indicating an increase in number of larvae with increase in concentration). One-way ANOVA indicated a significant difference between mean % larvae at different concentrations ($F = 31.9$, $p = 0.0001$). The post hoc Tukey's HSD test showed that there was no significant increase in attraction after the dose of 19.25 nl cm^{-2}. Interestingly, in this bioassay, data (figure 3) indicated a dose-dependent attractive property of farnesol, which is a usual response pattern for pheromone/pheromone-like compounds [13]. Our findings, therefore, indicate that the larval cuticle of *Chironomus* contains 'pheromone-like' compound(s).

Care has been taken to simulate the conditions found in the natural habitats of *Chironomus* larvae. These larvae are bottom dwellers, mostly in the shallow zones of water bodies. Under natural conditions, the larvae crawl on the substratum and make tubes out of sand, mud, or particulate detritus. Swimming is a secondary activity, and the larvae prefer to remain inside

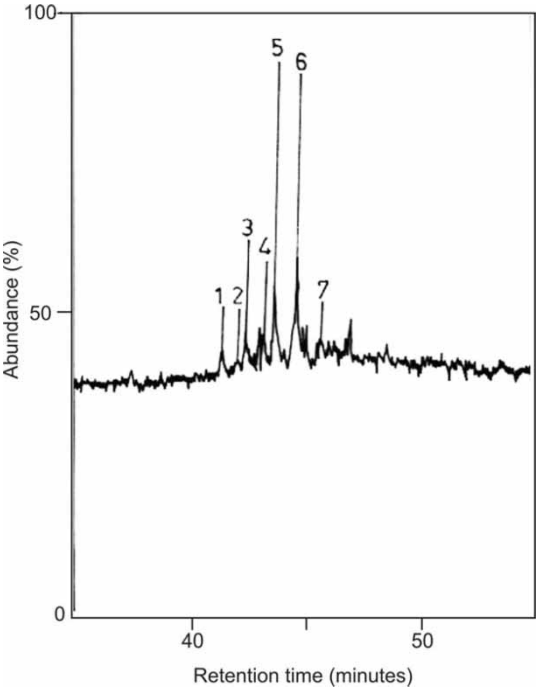

Figure 2. Gas chromatogram obtained by GC-MS analysis of a cuticular extract of *Chironomus* larvae showing retention time and percentage abundance.

Table 1. Signals obtained from the GC-MS analysis of the cuticular extract.

Sr. no.	Retention time (min)	Fragmentation pattern
1	41.3	69 (100), 93(72), 107 (56), 121 (48), 135 (44), 147(20), 159 (12), 175 (20), 190 (12), 201 (12)
2	42.15	69 (100), 93 (61.5), 107 (57.6), 121 (46.15), 35 (19.2), 145 (19.2), 159 (19.2), 175 (19.2), 185 (11.5), 205 (11.5)
3	42.72	69 (100), 91(66.6), 105 (71.42), 119 (61.9), 133 (52.2), 145 (42.8), 159 (47.6), 175 (52.3), 187(19), 201(28.5), 212 (9.5)
4	42.9	69 (100), 93 (51.72), 107 (48.2), 121 (34.4), 133 (31), 145 (20.6), 159 (17.2), 175 (24.1), 189 (6.8), 201 (10.3)
5[a]	43.28	69 (100), 81 (40.9), 91 (24.45), 105 (22.72), 121 (22.72), 135 (9.09), 145 (9.09), 159 (6.8), 173 (4.5), 189 (2.2), 203 (2.2)
6[a]	44.34	69 (100), 81 (24.39), 91 (24.39), 105 (24.39), 121 (29.26), 135 (9.75), 145 (7.31), 159 (7.31), 173 (4.87),185 (4.87), 199 (4.87)
7	45.4	69(100), 91(80), 107(60), 121 (65), 136 (60), 149 (45), 159 (30), 171(20), 185(20), 201(25)

Note: Figures in parentheses indicate the percentage of the fragment ion.
[a]Major signals as indicated by the area under the curve, identified as farnesol and farnesene respectively by NIST library.

the tubes. It can be speculated that the larval contact with the substratum might provide chemical cues for 'trails' in and around the tubes. Since the bioassay using the tube extract (data not presented) indicated no significant attractive properties, it is logical to hypothesize the presence of 'pheromone-like' compounds in the larval cuticle itself. Pheromonal activities of long-chain aliphatic compounds like (*Z*,*Z*,*E*)-3,6,8 trimethyl-dodecatrienol and terpenoides like (*Z*,*E*)-α-farnesene have been found in *Coptotemes* (Isopteran insect) and in *Solenopsis*

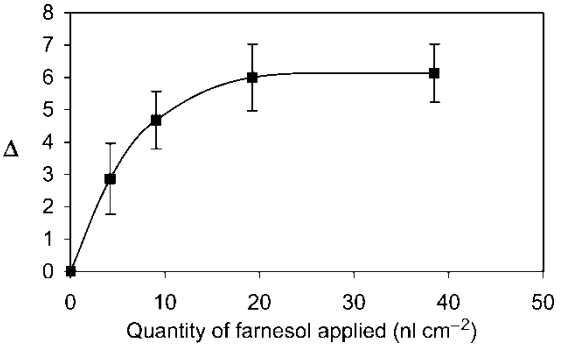

Figure 3. Graph of Δ, the difference in number of larvae (mean ± S.D.) attracted to the coated zone and uncoated zone, against the quantity of farnesol coated.

(Hymenopteran insect), respectively [14, 15]. The pheromonal activity of α- and β-farnesene has been reported in insects like honeybees [16], aphids [17], beetles [18], fruit flies [19], and even mammals [20]. It is also important to note that dodecatrienol is an analogue of farnesene [21].

The compounds responsible for chemical signalling, reported from non-arthropod aquatic organisms, are different from the known insect pheromones [22–24]. A few species of anuran frog and freshwater fish also emit peptide pheromone [25] as well as metabolites of steroidal hormones [26] in an aquatic environment as signalling compounds. Among the other invertebrates, the role of diterpene molecules in the inhibition of barnacle settlement was demonstrated by Clare *et al.* [27]. Painter *et al.* [28, 29] have reported the mate-attracting pheromone 'attractin' in *Aplysia* (Mollusc), which can emit long-distance water-borne signals. Similarly, Hardege *et al.* [30] have demonstrated that females of the ragworm, *Nereis succinea*, employ a tetra-peptide, cysteinyl-glutathione as a mate-recognition and gamate-releasing pheromone during reproduction. The role of a variety of eicosanoids and a number of trihydroxylated polyunsaturted fatty acid derivatives during the hatching of nauplii and the settlement of *Balanus amphritrite* cypris larvae has been demonstrated by Vogan *et al.* [31]. Although the mechanism of action of these compounds is as yet unexplained, they are likely to possess a pheromonal role.

A few reports of pheromones emitted by semi-aquatic insects are also worth mentioning. Löfstedt *et al.* [32] reported sex pheromones from different caddis flies, namely *Rhyacophila fasciata*, *R. nubila*, and *Hydropsyche augustipennis*, which are aquatic insects, but the pheromones were identified from the adults living in a terrestrial environment. The courtship behaviour of a semi-aquatic spider, *Dolomedes fimbriatus*, is presumably due to sex pheromones [33]. In *Culex quinquefasciatus*, an oviposition attractant pheromone is released by non-aquatic adults and is deposited on the egg-rafts above the water surface [34]. These reports do not claim for any water-borne pheromones, since these are deposited outside the waterbody. Nevertheless, in the literature, no report has yet been available on pheromones released by any aquatic stages of insect. Our findings, for the first time, have indicated the presence of 'pheromone-like' compounds in the aquatic larvae of *Chironomus* and have also confirmed the attractive properties of the cuticular extract. Further, results obtained from the bioassays have established the pheromonal activity of farnesol, one of the constituents identified from the cuticular extract.

It would be rather speculative to implicate constituents of the cuticular extracts in the 'tube-recognition behaviour' exhibited by the larvae [7], since we are in the early stages of understanding the role of these compounds in *Chironomus*. Nevertheless, our study will

encourage biologists to explore pheromone or pheromone-like compounds in other aquatic insects, which were hitherto unknown in the literature.

Acknowledgements

We thank Megha S. Nivsarkar and C. Joglekar, for their assistance in the initial part of the project, especially for the standardization of chemical extraction procedures with different solvents. One of the authors (BBN) acknowledges infrastructural facilities obtained from Department of Zoology (UGC-CAS and DST-FIST), University of Pune. Critical comments by Dr A. M. Diwan are gratefully acknowledged. We thank Mr Shashikant Arya, for his assistance in designing the illustrations. Thanks are due to Dr Shashi Chiplonkar, for the statistical analyses.

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