Chemical Ecology of Astigmatid Mites LXVII. Neral [3,7-dimethyl-(Z)-2,6-octadienal]: The Female Sex Pheromone of an Acarid mite, *Histiogaster* sp. (Acari: Acaridae)

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ABSTRACT

Neral [3,7-dimethyl-(Z)-2,6-octadienal] was identified to be the female sex pheromone of the mite *Histiogaster* sp. (Acari: Acaridae). The species originated from a fungal *Trichoderma harzianum* culture in Japan. The compound aroused males to mount females. Although the compound was detected in both sexes (29.4±8.9 ng in females and 5.7±1.9 ng in males), the behavior observation suggested that males could distinguish females from males. Synthetic neral indicated the activity at 0.1–1 ng doses. On the other hand, geranial [3,7-dimethyl-(E)-2,6-octadienal, the geometrical isomer of neral at the 2-C position], did not sexually stimulate males. Males could, therefore, discriminate between neral and geranial. Escaping behavior was observed upon exposure of neral at 100 ng and of geranial at 10–100 ng doses.

Key words: Astigmatid mite, *Histiogaster* sp., female sex pheromone, neral, 3,7-dimethyl-(Z)-2,6-octadienal

INTRODUCTION

Eight species of Astigmata are known to possess sex pheromones. These mites can be classified into two groups. *Acarus siro* (Levinson *et al.*, 1989) and *A. immobilis* (Sato *et al.*, 1993) belong to a group in which two sex pheromones, produced by each sex, are involved in their courtship rituals. The other group consists of species each possessing only the female sex pheromone, *Sancassania* (= *Caloglyphus*) *polyphyllae* (Leal *et al.*, 1989a), *Aleuroglyphus ovatus* (Kuwahara *et al.*, 1992), *S. rodriguezi* (Mori *et al.*, 1995), *Sancassania* sp. (tentatively named as *Sancassania* sp. MJ) (Mori *et al.*, 1996), another *Sancassania* sp. (tentatively named as *Sancassania* sp. HP) (Mori *et al.*, 1998), and *Cosmoglyphus hughesi* (Ryono *et al.*, 2001). Although these female sex pheromones arouse male's mounting behavior, males also contain more or less of these compounds. Their ratios between sexes (female/male) differ by species, and these ratios appears to be a key factor to differentiate whether males of a species can discriminate females from males or not (Mori and Kuwahara, 2000; Ryono *et al.*, 2001). Males selectively mount their females in the following cases: *Sancassania* sp. HP (ratio of female/male, 8.4/1), *S. rodriguezi* (6.3/1) and

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S. polyphyllae (3.4/1), while males cannot discriminate in the following cases: *Sancassania* sp. MJ (1.4/1) and *C. hughesi* (2.1/1).

As a part of the pheromone study on astigmatid mites, we examined the sex pheromone of *Histiogaster* sp., one of fungivorous species. The genus contains a number of species associated with subcortical habitats and fungal fruiting bodies. Several *Histiogaster* spp. are found in North America from elms and pine trees associated with weevils, bark beetles and long-horned beetles, and some of these mites use beetles for dispersal at the stage of deutonymphs and maybe eat either nematodes, fungi or the beetles (the eggs, larvae and pupa of them) (Jacot, 1936; Nesbitt, 1945; Griffiths, 1963; Davis and Hunter, 1963; Woodring, 1963, 1966; Hajek and Dahlsten, 1985; Kinn, 1987). The mite *Histiogaster* sp. used in this study originated from a fungal *Trichoderma harzianum* culture on July 1990 in Oita Prefecture (Japan). It is the first description of this genus in Japan (Okabe, 1993a). The species indicated an exponential population growth in feeding on several fungi except shiitake mushroom *Lentinula edodes*, and was suspected as possibly becoming a serious pest or an important pathogen vector for mushroom production (Okabe, 1993a, 1993b, 1994, 1996, 1999).

In the present paper, we identified the female sex pheromone of *Histiogaster* sp. The compound triggered mounting behavior of males. Based on its content difference between sexes, the male's ability to discriminate females from males was also discussed.

MATERIALS AND METHODS

Mites. A colony of the mite *Histiogaster* sp. (Acari: Acaridae) was maintained in a Petri dish (90 mm in diameter×20 mm in height) by feeding dried yeast at room temperature under humid conditions in our laboratory. The container was kept in a zip-lock plastic bag (240×170×0.04 mm) to maintain moisture level. Both sexes were randomly collected from the stock culture by a needle, and used without prior knowledge of their mating experiences. This strain was collected from a laboratory culture of the fungus *Trichoderma harzianum*, July 1990, in Oita Prefecture, Japan (Okabe, 1993a). *Trichoderma harzianum* is a serious economic pathogen for mushroom production.

Instrumental analyses. Gas chromatographic (GC) analysis was performed with a Hewlett Packard 6850 gas chromatograph with a flame ionization detector, using an HP-5 capillary column (0.25 mm×30 m, 0.25 µm in film thickness, Hewlett Packard) with helium as the carrier gas at 1.23 ml/min in the split-less mode at a temperature programmed from 60°C for 2 min to 290°C at 10°C/min and then held for 5 min. The chromatogram was processed with an HP-3395 integrator. A gas chromatograph/mass spectrometric (GC/MS) analysis was carried out using a Hewlett Packard HP-5890 GC/MS operated at 70 eV in the split-less mode, using the same column and temperature conditions mentioned above.

Extraction method. A group of ten males or females was separately placed in a conical tube (handmade, 8 mm in diameter×30 mm in height), and dipped in hexane (4 µl) for 3 min. The hexane layer was then taken up with a micro syringe (10 µl volume, Hamilton) and subjected to the GC/MS analysis.

For evaluating pheromonal activity, two females or males were extracted by dipping into hexane (100 µl) for 3 min. One microliter portion of the extract (corresponding to 0.02

female or male equivalents) was assayed against males or females.

To determine the pheromone content in each sex, one female or one male was extracted at a time for 3 min in hexane (10 μ l for female and 5 μ l for male), containing tetradecane (2 ng/ μ l) as an internal standard. One microliter portion of each hexane extract corresponding to either 0.1 female or 0.2 male equivalents was analyzed by GC.

To determine the active compound(s), one hundred female mites were separated from the stock culture, and extracted by dipping in hexane (100 μ l) for 3 min. The extract was, without concentration, subjected to a conventional silica gel column chromatography (Wakogel C-200, 800 mg), and the column was eluted with 5 ml each of the following solvents: hexane, a mixture of ether in hexane (10%, 20% and 50%), and ether. Then, each fraction (1 μ l portion, corresponding to 0.02 female equivalents) was subjected to bioassay. The remaining portions of each fraction were analyzed by GC/MS.

Biological assays. The bioassay was conducted in a small Petri dish (8 mm in diameter $\times$ 5 mm in height), whose bottom surface was covered with a damp filter paper disc (8 mm in diameter). To the dish, ten males (or females) were introduced with a small amount of moistened dry yeast, and the dish was closed with a cover glass to prevent the mites from escaping. After 3–5 hours of conditioning, a female or a male was introduced at the center of the chamber to determine or to detect the presence of the pheromone. Similarly, a piece of filter paper (2 mm $\times$ 2 mm) impregnated with the following candidate materials at the indicated doses was introduced: either female-, male-extract, column fractions or synthetic compounds (neral or geranial at 0.01, 0.1, 1, 10 and 100 ng/ μ l dose in hexane). A piece of filter paper impregnated with hexane was used as control. The number of mounting attempts by males was counted for 5 min under a binocular microscope. The assay was conducted at 23°C with repetition of 5–10 times in each case.

Preparation of neral and geranial. Neral (purity more than 97%) and geranial (purity more than 99%) were each prepared from nerol and geraniol as previously reported (Leal *et al.*, 1989d).

Data analyses. The pheromone content of both sexes was analyzed by the Mann-Whitney *U* test. For the bioassay, the results were processed to evaluate significant differences in the numbers of mounting attempts by the Mann-Whitney *U* test or the Kruskal-Wallis test followed by the Dunn's multiple-comparison test.

RESULTS

Hexane extracts of both sexes commonly showed the following six peaks by GC/MS analyses with each quantitative difference; peak A (t_R =9.30 min), peak B (t_R =9.49 min), peak C (t_R =9.90 min), peak D (t_R =10.06 min), peak E (t_R =10.28 min), peak F (t_R =11.02 min) (Fig. 1). Peak B was the major component in females, while it was the second major in males. The molecular and diagnostic ions of each peak are summarized in Table 1. Based on their mass spectra and GC retention times (t_R) together with those of authentic compounds, all the peaks (A–F) were identified to be nerol [3,7-dimethyl-(*Z*)-2,6-octadienol] (Kuwahara and Suzuki, 1983), neral [3,7-dimethyl-(*Z*)-2,6-octadienal], geranial [3,7-dimethyl-(*E*)-2,6-octadienal] (Kuwahara *et al.*, 1980a), α -acaridial [(*E*)-2-(4-methyl-3-pentenyl)-butenedial] (Leal *et al.*, 1989c), tridecane (Howard *et al.*, 1988) and β -acaridial

Fig. 1. Typical gas chromatograms of the hexane extracts from *Histogaster* sp. (I) 10 female equivalents and (II) 10 male equivalents. Chromatograms were obtained with an HP-5 capillary column (0.25 mm×30 m, 0.25 μ m in film thickness, programmed from 60°C for 2 min to 290°C at 10°C/min and then held for 5 min).

Table 1. GC/MS Data for Compounds Obtained from the Hexane Extract of *Histogaster* sp. GC Retention Times, Molecular and Diagnostic Ions, and Relative Abundance

Compound (peak A–F)	GC t_R (min)	Relative abundance (%)		Molecular and diagnostic ions m/z (intensity %)
		Female	Male	
A: Nerol	9.30	11.2	1.9	41 (68), 69 (100), 84 (20), 93 (58), 121 (23), 136 (17), 154 (M^+ , 5)
B: Neral	9.49	37.9	22.1	41 (90), 69 (100), 84 (34), 94 (46), 109 (32), 119 (20), 134 (12), 137 (8), 152 (M^+ , 3)
C: Geranial	9.90	3.8	1.9	41 (69), 69 (100), 84 (30), 94 (22), 109 (10), 123 (9), 137 (13), 152 (M^+ , 9)
D: α -Acaridial	10.06	6.0	5.6	41 (100), 69 (92), 95 (15), 108 (17), 119 (15), 123 (26), 133 (25), 151 (12), 166 (M^+ , 10)
E: Tridecane	10.28	29.4	47.7	43 (66), 57 (100), 71 (73), 85 (49), 99 (14), 113 (9), 127 (9), 141 (7), 155 (4), 184 (M^+ , 24)
F: β -Acaridial	11.02	11.7	20.8	41 (100), 55 (48), 67 (96), 81 (54), 95 (73), 108 (44), 123 (65), 133 (68), 151 (42), 166 (M^+ , 25)

[(*E*)-2-(4-methyl-3-pentenylidene)-butanedial] (Leal *et al.*, 1989b), respectively.

When a female was introduced to an assay chamber containing conditioned males, the males started to mount the introduced female and/or other males. The average count of

mounting attempts during the testing period was observed to be 10.3 ± 0.4 ($n=10$). Although the same behavior was observable by a male introduction to the chamber, the count was significantly less frequent, 6.5 ± 0.5 ($n=10$) on average. There was a significant difference between these two cases (Mann-Whitney U test, $p < 0.01$), and therefore, the males could discriminate the females from the males.

Upon exposure of a piece of filter paper impregnated with 0.02 equivalent of female or male extract, the tested males were aroused sexually and mounting behavior was also observable. The resulting mounting frequency was 8.1 ± 0.6 ($n=10$) on average for the female extract, while it was 5.8 ± 0.3 ($n=10$) on average for the male extract and 3.5 ± 0.2 ($n=10$) for the hexane impregnated control (Fig. 2). Significant differences were demonstrated between mite extracts and control (Kruskal-Wallis test followed by the Dunn's multiple-comparison test, $p < 0.01$ for the female extract and $p < 0.05$ for the male extract). The presence of the female sex pheromone was, therefore, confirmed not only by a female alone but also by the hexane extract. There was no significant difference observed between the extracts from females and males (Kruskal-Wallis test followed by the Dunn's multiple-comparison test, $p > 0.05$), although the female extract triggered more frequent mounting attempts than the male extract.

The 10% ether in hexane fraction obtained by a silica gel column using the hexane extract from 100 females was concluded to be active by bioassay (Fig. 3), and the fraction consisted of a mixture of neral and geranial by GC/MS analyses (Fig. 3). Either geranial or neral might, therefore, be the female sex pheromone.

The dose-response relationship of synthetic neral indicated a convex curve with the maximum activities at 0.1 and 1 ng-dose within the range examined from 0.01 ng to 100 ng

Fig. 2. Sex pheromone activity of the hexane extracts from *Histiogaster* sp. 0.02 female equivalents and 0.02 male equivalents (* $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test followed by Dunn's multiple-comparison test, each $n=10$).

Fig. 3. Purification procedure to the hexane extract of mites and the sex pheromone activity of each fraction at 0.02 female equivalents (** $p < 0.01$, Kruskal-Wallis test followed by Dunn's multiple-comparison test, each $n = 10$).

Fig. 4. Sex pheromone activity of synthetic neral (* $p < 0.05$, Kruskal-Wallis test followed by Dunn's multiple-comparison test, each $n = 10$).

(Fig. 4). Significant differences were demonstrated between control and either 0.1 or 1 ng-dose (Kruskal-Wallis test followed by the Dunn's multiple-comparison test, $p < 0.05$). On the other hand, synthetic geranial did not sexually stimulate males: the resulting mounting frequencies were 3.2 ± 0.4 , 3.2 ± 0.4 , 3.8 ± 0.4 , 0.8 ± 0.6 , 0.0 ± 0.0 (each case, $n = 5$) for 0.01, 0.1,

1, 10 and 100 ng doses, respectively. As a result, neral was concluded to be the female sex pheromone. The pheromone activity was lost at a 10 ng dose of neral, and males escaped from the test filter paper at 100 ng dose. The same escaping behavior was observed when 10–100 ng of geranial was introduced.

The pheromone neral was contained as the major component in females at 29.4 ± 8.9 ng ($n=5$) on average, whereas it was the second major in males at 5.7 ± 1.9 ng ($n=5$) among six components detected by GLC (Fig. 1 and/or Table 1). The pheromone content ratio of females to males is ca. 5.2/1. This represents a significant difference between sexes (Mann-Whitney U test, $p < 0.05$).

DISCUSSION

Neral (active at 0.1–1 ng doses) was identified to be the female sex pheromone of the mite *Histiogaster* sp., although the compound was present not only in females (29.4 ± 8.9 ng) but also in males (5.7 ± 1.9 ng), as in the cases of other astigmatid mites (Mori and Kuwahara, 2000). This is consistent with the fact that both extracts from each sex demonstrated the activity at 0.02 mite equivalents, which corresponded to ca. 0.6 ng and ca. 0.1 ng of neral. The same mounting behavior was observed when a male or a female was introduced to the assay chamber containing conditioned males, although a higher frequency was recorded upon a female introduction (10.3 ± 0.4) than a male introduction (6.5 ± 0.5). This implies that a male could distinguish females from males for mounting and mating. In fact, mountings (and matings) were exclusively observable between female and male combinations in the stock culture, and homosexual mounting took place only in the assay chamber. It was also often noticeable in the stock culture that a female was carrying more than two males competing with each other on her abdomen. This male preference toward females was explainable by the content ratio of the pheromone (5.2/1). Species with a ratio of more than 3.4/1 are classified as the ones whose males can discriminate sexes and select a mate among the genus *Sancassania* (= *Caloglyphus*) (Mori and Kuwahara, 2000).

The sex pheromone activity of synthetic neral was lost at a 10 ng dose, whereas escaping behavior was observed at a 100 ng dose not only among males but also females. This 100 ng of neral corresponds to 3.4 female equivalents, and implies the possibility of an alarm pheromone function in the species. Recently, the sex pheromone of *S. polyphyllae*, β -acaridial has also been found to function as an aggregation pheromone (Shimizu *et al.*, 2001), and the alarm pheromone neral in *Schwiebea elongata* has also been found to function as the attractant (Nishimura *et al.*, 2002). Further study will be needed to conclude the presence of the alarm pheromone in the present species, because mites near to disturbed or squashed ones did not unambiguously escape in the stock culture, and squashed mites were often eaten by ones nearby. There is, however, a small possibility that mites may start escaping when a large number of mites are squashed at the same time in the natural environment.

On the other hand, geranial, the geometrical isomer of neral at the 2-*C* position, exhibited no sexual activity within the same dose range examined (0.01–100 ng) as neral. The results suggested the male's ability to discriminate the geometrical isomer. Upon exposure to a 10- and 100-ng dose against both sexes unexpectedly displayed the escaping behavior. This fact, however, may not be important as a biological function, because geranial was the most

minor component in the extract from both sexes.

Neral is one of the most widely distributed components among astigmatid mites, and has been detected in 31 species among the 52 species investigated (Kuwahara, 1999). Five species use the compound as their alarm pheromone: *Carpoglyphus lactis*, *Lardoglyphus konoi* (Kuwahara *et al.*, 1980b), *Suidasia medanensis* (Leal *et al.*, 1989d), *Glycyphagus domesticus* (Kuwahara *et al.*, 1991), *Schwiebea elongata* (Kuwahara *et al.*, 2001). However, this is the first example identifying the function of neral as the female sex pheromone. It is also the first identification of the female sex pheromone among species belonging to the genus *Histiogaster*.

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摘 要

ネラール：コナダニ科 *Histiogaster* sp. 雌性フェロモンの同定 平岡弘之・森 直樹・西田律夫・桑原保正（京大院農）・岡部貴美子（森林総研）

ネラール（3,7-ジメチル-(Z)-2,6-オクタジエナール）を *Histiogaster* 属未同定種の雌の性フェロモンとして同定した。このダニは椎茸栽培時に発生した病原糸状菌 *Trichoderma harzianum* の菌そうから採集された種である。ネラールは雄ダニを性的に興奮させ、定量すると雌には 29.4 ± 8.9 ng、雄には 5.7 ± 1.9 ng 含まれていた。他の無気門亜目ダニと同様にフェロモンは雄ダニにも存在したが、行動観察から雄ダニは雌ダニを識別できると判断した。合成化合物は $0.1-1$ ng で活性を示した。一方幾何異性体のゲラニアール（3,7-ジメチル-(E)-2,6-オクタジエナール）に活性は認められず、雄はネラールとゲラニアールを区別できることが判明した。ネラールを 100 ng、ゲラニアールを $10-100$ ng 投与するとダニは忌避行動を示した。