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Aggregation Pheromone Activity of the Female Sex Pheromone, β-Acaridial, in *Caloglyphus polyphyllae* (Acari: Acaridae)†

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***Caloglyphus* (=*Sancasania*) *polyphyllae* discharges from a pair of opisthonotal glands a characteristic set of volatiles, *i.e.* three monoterpenes and seven hydrocarbons. Among them, β-acaridial, which is known as the female sex pheromone of the species and has antifungal activity, was newly identified as the aggregation pheromone for unfeeding and unmating mites. Feeding mites, however, exhibited sexually aroused behavior instead of the tendency to cluster when exposed to β-acaridial. This is the first example of the compound demonstrating two pheromone functions depending upon the circumstances faced by the mites.**

Key words: mite; *Caloglyphus polyphyllae*; β-acaridial; aggregation pheromone; sex pheromone

Phoretic deutonymphs of the species *Caloglyphus* (=*Sancasania*) *polyphyllae* Zakhvatkin 1941 (Astigmata: Acaridae) are closely related to *Polyphyllae laticollis* Lewis (Coleoptera: Scarabaeidae) which is a rare species only collectable in mountainous regions of Japan. The mites are collected from underneath the beetle's elytra as those deutonymphs. Although the species seems to be a scavenger in the natural environment, and is a possible pest for agriculture, there are no records available of its adult collection in the natural habitat.

β-Acaridial [2(*E*)-(4-methyl-3-pentenylidene)-butanedial],[1] a component emitted from a pair of opisthonotal glands, has been identified as the female sex pheromone of the species. This is the first example of the female sex pheromone being identified among astigmatid mites. The glands have recently been realized to contain at least three more compounds whose structures remain unknown, along with the following 8 compounds: rosefuran, α-acaridial [2(*E*)-(4-methyl-3-pentenyl)-butenedial], (*Z*,*Z*)-6,9-heptadecadiene, (*Z*)-8-heptadecene, (*Z*)-7-pentadecene, heptadecane, pentadecane, and tridecane (Fig. 1). Reinvestigation of the gland components has resulted in the identification of three more new compounds: hydrocarbon (*Z*,*Z*)-1,6,9-heptadecatriene[2] and two monoterpenes (*m*/*z* 168A and *m*/*z* 168B, manuscript in preparation).

Aggregation has often been noticed in the usual rearing medium. Although part of this aggregation phenomenon might be attributable to the mite's inclination toward such physical factors as darkness, roughness and the microclimate of the spots, the

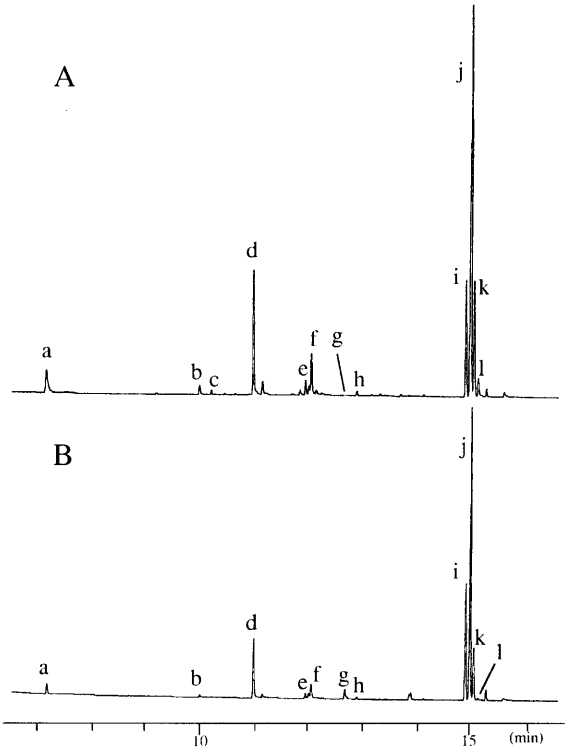

Fig. 1. Typical Gas Liquid Chromatograms of Extracts from 3 Female Equivalents (A) and 3 Male Equivalents (B) of *Caloglyphus polyphyllae*.

a, rosefuran; b, α-acaridial; c, tridecane; d, β-acaridial; e, M168B; f, M168A; g, *Z*-7-pentadecene; h, pentadecane; i, *Z*,*Z*-1,6,9-heptadecatriene; j, *Z*,*Z*-6,9-heptadecadiene; k, *Z*-8-heptadecene; l, heptadecane.

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same behavior has also been observed when the mites were introduced into a new and non-accustomed place. These phenomena imply the presence of an aggregation pheromone in the species.

We report here the identification of the aggregation pheromone which triggers mites' aggregation behavior not only among both sexes of the species, but also at all developmental stages. The active principle of the pheromone was found to be the same as that of the female sex pheromone. We also discuss how the compound functions as a female sex pheromone and as an aggregation pheromone under different conditions.

Materials and Methods

Mite. Caloglyphus polyphyllae Zakhvatkin subdivided by Dr. Kazuyoshi Kurosa (Tokyo) originated from hypopi obtained from *Polyphyllae laticollis* Lewis (Coleoptera: Scarabaeidae) which had been captured on 4th August 1994 from Mt. Odamiyama in Ehime prefecture. The species was maintained at 20–25°C by feeding on dry yeast in a plastic Petri dish (85 mm i.d., 20 mm depth) whose base was covered with a sheet of moistened filter paper to maintain high humidity. Each dish was kept in a zip-lock polyethylene bag (240 × 170 mm, 0.04 mm film thickness). Both sexes for behavioral experiments were randomly sampled from the stock culture.

Analytical method. Gas liquid chromatography (GC) was conducted with a Hewlett Packard 5890 series II Plus equipped with FID and an HP-5 capillary column (0.25 mm i.d. × 30 m, Hewlett Packard, split-less mode) at a temperature programmed from 60°C (with an initial 2-min hold) to 290°C (with a 5-min hold) at 10°C/min. Gas liquid chromatography coupled with mass spectrometry (GC/MS) was carried out by a Hewlett Packard HP-5890 gas chromatograph-mass spectrometer operated at 70 eV in the split-less mode, using an HP-5 MS capillary column (0.32 mm × 30 mm, 0.33 μm film thickness) under the same conditions as those just stated.

Quantitative determination of the pheromone. Two males or two females at a time were collected by a needle into a small conical-bottomed tube (handmade, 8 mm o.d. × 30 mm height.). Hexane (4 μl) containing hexadecane (80 ng) as an internal standard was added to each tube for extraction. After 3 min, a 1-μl portion of the extract corresponding to 0.5 male or female equivalent was subjected to a GC analysis. The quantity of β-acaridial per male or female was determined by the relative ratio of the peak area to that of the internal standard with 10 replicates.

Extraction and purification of the pheromone. Mites (2.0 g) at all developmental stages of both sexes, after being separated from the culture medium by the conventional saline flotation method, were dipped into hexane (5 ml) for 3 min. The resulting hexane extract (18 mg), after evaporating the solvent, was applied to a silica gel column (500 mg, Wako-gel C-200), which was eluted by 5 ml each of the following solvents: hexane, hexane-ether (20:1), (10:1), (5:1) and (2:1), and ether. The activity and composition of each column eluate was monitored by a bioassay, GC and GC/MS.

Bioassay method. Three bioassay methods were applied, using the same size of plastic Petri dish (85 mm i.d., 20 mm depth) as an assay chamber whose base was covered with a piece of moistened filter paper. In method 1, test mites (a group of fifteen males and fifteen females, thirty males or thirty females) collected from the stock culture were introduced to the chamber, and a piece of filter paper (5 × 5 mm), which had been impregnated with a candidate sample such as 2 μl of the whole-mite extract (5 ml) or each separated fraction (5 ml), was gently placed in the center of the chamber. In method 2, fifteen paired couples were also placed in the chamber and then a filter paper impregnated with a candidate sample was introduced into the chamber. Method 3 used a mixture of fifteen males and fifteen females placed in the chamber with a small amount of moistened dry yeast as the foodstuff. After being conditioned for 30–60 min in the Petri dish and confirming that all mites were at the food, a piece of filter paper impregnated with a candidate sample was then introduced to the center of the chamber. With all three methods, the number of mites attracted toward the filter paper was counted for 5 min under a stereoscopic microscope after the introduction of each sample, and the results were evaluated as percentages. Hexane was used as the control. The aggregation pheromone activity was also compared among methods (1–3) by using fifteen males and fifteen females, tests being conducted at room temperature under ambient humidity. All bioassays were repeated 5–10 times, and the results obtained were processed to evaluate significant differences by a Mann-Whitney *U* test or a Kruskal-Wallis test followed by Dunn's multiple-comparison test.

Preparation of β-acaridial. β-acaridial was synthesized by starting from 1,2,4-butanetriol and 1-bromo-4-methyl-3-pentene. Experimental details for the preparation of β-acaridial will be reported elsewhere. ^{1}H-NMR ($CDCl_3$) spectral data are as follows: δ 1.65 and 1.70 (3H each, s), 3.00 (2H, dd, J= 7.29 and 7.30 Hz), 3.43 (2H, d, J=1.3 Hz), 5.12 (1H, m), 6.75 (1H, t, J=7.44 Hz), 9.45 (1H, s) and 9.62 (1H, t, J=1.6 Hz).

Results

Identification of the aggregation pheromone

When a mite group composed either of both sexes, of all males or of all females was exposed to a piece of filter paper impregnated with the crude hexane extract by method 1, the mites came close to the filter paper and stayed around it for a while. Except for a case of the female's chamber, copulation behavior (hetero-sexual or homo-sexual) with mounting attempts was occasionally observed near the filter paper after being attracted, not only on the introduced filter paper or around it, but also within a close range. The percentages of this aggregation in the three assay chambers (both sexes, males, and females) for 5 min of observation were respectively $81.2 \pm 1.5\%$, $82.2 \pm 3.7\%$ and $83.1 \pm 2.0\%$ (each $N = 10$, Kruskal-Wallis test followed by Dunn's multiple-comparison test, $p < 0.01$ against the control) (Fig. 2). As a result, no sexual differences were apparent with the aggregation behavior of each tested mite group.

The hexane extract from both sexes was chromatographed in an SiO_2 column eluted stepwise with hexane-ether mixtures. The aggregation activity of the extract was recovered in fractions eluted with hexane-ether 10:1 and 5:1 (Fig. 3). All the other fractions were entirely inactive. The active hexane-ether (10:1) fraction by GC/MS produced two peaks, these being identified as α-acaridial (t_R 10.07 min) and β-acaridial (t_R 11.04 min). The other active fraction (5:1) produced one peak which was identified as β-acaridial. The aggregation rates for a test chamber of both sexes were $70.5 \pm 3.5\%$ and $88.0 \pm 2.4\%$ (Kruskal-Wallis test followed by Dunn's multiple-comparison test, each $p < 0.01$) for the 10:1 and 5:1 fractions. β-acaridial was the common compound in both active fractions and, therefore, seems to have been the active principle.

Evaluation of the synthetic aggregation pheromone

The dose-response relationship for synthetic β-acaridial showed a convex curve and gave the optimum range as summarized in Fig. 4. Results (dose, %, each $N = 10$) were as follows: 0.1 ng, 47.2 ± 6.8; 1 ng, 78.0 ± 4.8; 10 ng, 86.5 ± 1.9; 100 ng, 74.5 ± 5.1; and 1000 ng, 21.1 ± 1.7. The doses of 1 and 10 ng were statistically significant at $p < 0.05$ and $p < 0.01$, respectively. All the other doses were not significant against the control. As a result, β-acaridial was identified as the aggregation pheromone.

Quantitative determination of the pheromone

β-Acaridial was distributed in all developmental stages and was one of the major components. Its content was determined by following the method described above. Males contained 1.2 ± 0.3 ng on average, as the third major component (the most major component was (*Z*,*Z*)-6,9-heptadecadiene; the next major one was (*Z*,*Z*)-1,6,9-heptadecatriene). On the other hand, females contained 2.0 ± 0.3 ng of β-acaridial on average as the second major component (after (*Z*,*Z*)-6,9-heptadecadiene). The female/male ratio was 1.7 (see Fig. 1).

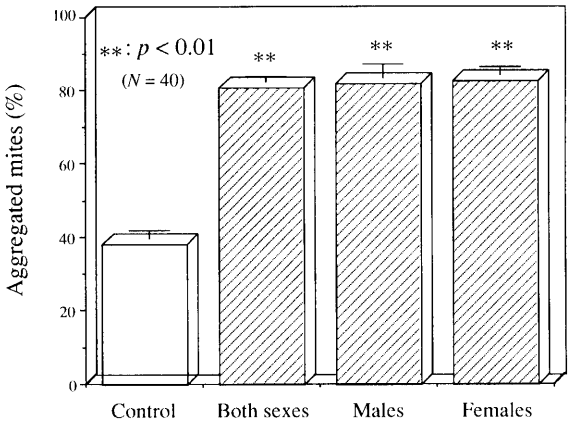
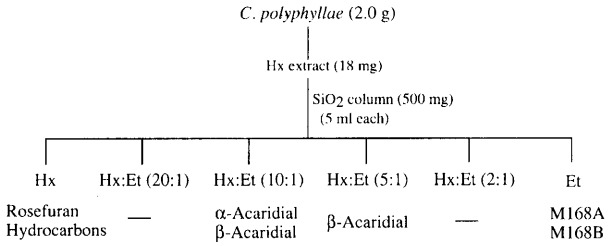


Fig. 2. Aggregation Pheromone Activity of the Whole-mite Hexane Extract by Bioassay Method 1.
Significant differences were determined by a Kruskal-Wallis test followed by Dunn's multiple-comparison test.



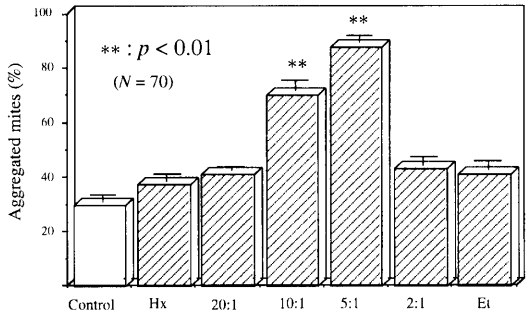


Fig. 3. Purification Procedure to the Hexane Extract of Mites and the Aggregation Pheromone Activity of Each Fraction by Bioassay Method 1.
Significant differences were determined by a Kruskal-Wallis test followed by Dunn's multiple-comparison test (Hx: hexane; Et: diethyl ether).

Comparison of the aggregation pheromone activity by the three bioassay methods (1-3)

Bioassay method 1 resulted in the test mites (a group of both sexes) being intensely attracted to the filter paper impregnated with β-acaridial (10 ng), the

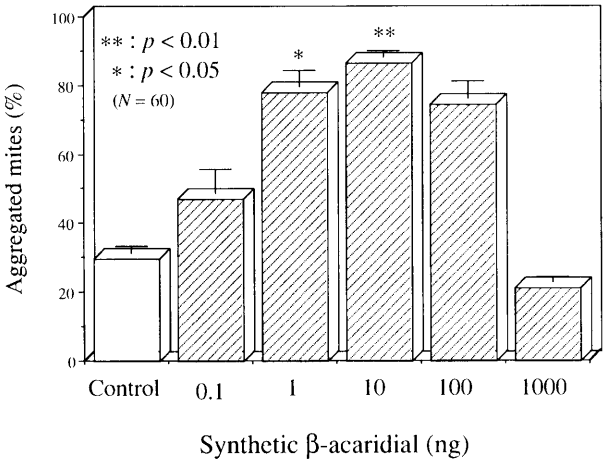


Fig. 4. Aggregation Pheromone Activity of Synthetic *β*-Acaridial by Bioassay Method 1.
Significant differences were determined by a Kruskal-Wallis test followed by Dunn's multiple-comparison test.

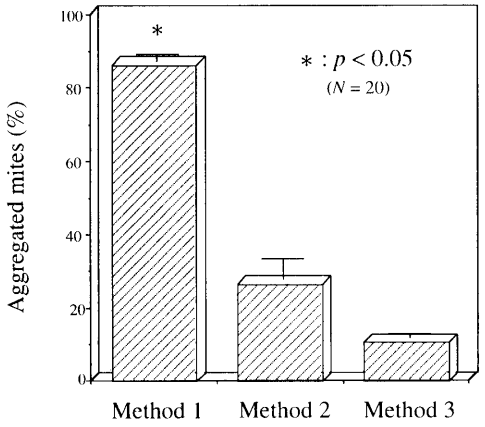


Fig. 5. Comparison of the Aggregation Pheromone Activity among Bioassay Methods 1-3.
Significant differences were determined by a Kruskal-Wallis test followed by Dunn's multiple-comparison test.

aggregation rate being 86.5±1.9% ($N=10$). On the contrary, mites copulating with method 2 were rarely attracted to the same filter paper used in method 1 (26.6±5.6%, $N=5$) and there was no significant difference apparent against the control. Method 3 resulted in a greater part of the test mites crowding near the foodstuff, regardless of the introduction of the filter paper impregnated with 10 ng of *β*-acaridial (10.7±1.2%, $N=5$), and a number of male mites in the chamber containing both sexes were observed to start homo- and hetero-sexual mounting behavior as reported by Leal *et al.*[1] This case also did not indicate any significant difference against the control. When the aggregation pheromone activity was compared among bioassay methods 1-3, the results of method 1 indicate activity ($p<0.05$, Fig. 5), while method 3 indicates sex pheromone activity instead of aggregation pheromone activity. The aggregation pheromone was thus ineffective with copulation or while eating food, and the sex pheromone activity by which male mites were sexually aroused appeared under these conditions.

Discussion

Aggregation pheromones or assembling pheromones are distributed in many species of animals such as insects[3] and ticks,[4] while only one species, *Lardoglyphus konoi*,[5] is known to possess the pheromone in acarid mites. This species is exceptional among Astigmata by possessing two pheromones: the alarm pheromone, citral,[6] and the aggregation pheromone, (*R*,*R*,*R*,*R*)-1,3,5,7-tetramethyldecyl formate.[7] At present, no other astigmatid species is known to possess two pheromones, because it is rational to consider that only one excretory gland (a pair of opisthonotal glands) is available in astigmatid mites, and all pheromones so far identified are compounds contained in these glands. In fact, all of the other astigmatid mites possess only one kind of pheromone, if any: the alarm pheromone or the sex pheromone.

The present study demonstrates that *Caloglyphus* (=*Sancasania*) *polyphyllae* possessed two kinds of pheromone (the aggregation pheromone and the female sex pheromone) which were evoked by a compound (*β*-acaridial) and manifested depending upon the mite's condition. This is the second example of a species possessing two kinds of pheromone, and the first example of a single compound with dual functions. More examples of the multi-pheromone system can be expected among Astigmata if we can explain how mites manipulate multi-functional chemicals by using only one excretory gland. Since mites are gregarious in nature, it is not surprising for them to handle more than one kind of semiochemical.

Sex pheromones are known in four *Caloglyphus* species (Acari: Acaridae): *β*-acaridial [2(*E*)-(4-methyl-3-pentenylidene)-butanedial] in *Caloglyphus polyphyllae*,[1] undecane in *Caloglyphus rodriguezi*,[8] (2*R*,3*R*)-epoxyneral [(2*R*,3*R*)-2,3-epoxy-3,7-dimethyl-6-octenal] in *Caloglyphus* sp. MJ,[9] and rosefuran in *Caloglyphus* sp. HP.[10] *Caloglyphus polyphyllae* is the first astigmatid mite whose female sex pheromone has been identified. Although the female/male ratio for *β*-acaridial content is known, the actual content of *β*-acaridial has not previously been reported.[11] It was evaluated in the present study by GC as 2.0±0.3 ng on average for females and 1.2±0.3 ng on average for males.

Behavioral observation of the species indicated that mounting activity was evoked more often after the male mites had been attracted to the aggregation pheromone (bioassay method 1), and after male mites had been exposed to the pheromone while eating food (bioassay method 3). No specific pattern of behavior was, however, apparent among mating

males upon exposure to the pheromone by method 2. No females showed a particular pattern of behavior in bioassay methods 2 and 3. However, under unconditioned circumstances (bioassay method 1), β-acaridial was responsible for attracting not only males but also females and mites at other developmental stages (data not shown), thus functioning as an aggregation pheromone. The aggregation pheromone activity is thought to have been weaker than starvation or sexual desire[4] which is why the aggregation pheromone activity was not revealed by bioassay methods 2 and 3.

These facts imply that β-acaridial functions as the aggregation pheromone in an unconditioned situation such as just after introduction to a new place, and then functions as the female sex pheromone under such circumstances as after aggregation and while eating food. In other words, the aggregation pheromone first works to assemble individual mites in a certain place, and then the female sex pheromone activity is manifested in the cramped conditions. If such a sequential role shift takes place between two pheromones, it may be reasonable to propose that the primary function of the compound is aggregation, which is why the pheromone is distributed almost evenly between both sexes. It is also rational to consider that the sex pheromone of the species is at an evolutionally primitive stage, as has been suggested from a COI gene comparison among three *Caloglyphus* spp,[12] because the compound should be shared equally between sexes to effectively function as an aggregation pheromone.

The species thrives in the nature by consuming patchily distributed food sources. If mites leave their foodstuff region, their lives might not be sustainable. The aggregation pheromone, therefore, might be an indispensable principle to increase the chance of mating, and to maintain suitable humidity in clusters.

Acknowledgment

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