Identification of Neryl Formate as the Airborne Aggregation Pheromone for the American House Dust Mite and the European House Dust Mite (Acari: Epidermoptidae)

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ABSTRACT   The American house dust mite, *Dermatophagoides farinae* Hughes, and European house dust mite, *Dermatophagoides pteronyssinus* Trouessart, are major pests of medical importance throughout the developed world, causing atopic diseases such as asthma, rhinitis, and atopic dermatitis. Previous studies in our laboratory have shown that the behavioral responses of house dust mites toward volatiles from food sources could be assessed using a Y-tube olfactometer assay. The current study used this Y-tube assay to investigate house dust mite pheromones. A hexane extract of *D. farinae*, along with fractions of the extract prepared by microscale liquid chromatography over Florisil, were tested for behavioral activity. One of the chromatographic fractions was shown to be significantly attractive ($P < 0.05$) for *D. farinae*, compared with a solvent control. Coupled gas chromatography-mass spectrometry analysis of this behaviorally active fraction indicated that neryl or geranyl formate was the major component. Peak enhancement by gas chromatography, using authentic samples of the neryl and geranyl isomers prepared in high purity by chemical synthesis, confirmed the identity of the major peak as neryl formate. In Y-tube assays, male and female *D. farinae* and *D. pteronyssinus* both were significantly attracted to synthetic neryl formate at doses of 100 and 10 ng, respectively ($P < 0.05$). No significant differences were found for *D. farinae* and *D. pteronyssinus* when synthetic neryl formate and house dust mite extracts containing natural neryl formate were tested at the same level. Dynamic headspace collection of *D. farinae* and *D. pteronyssinus* colonies showed that neryl formate was released as a volatile organic compound by both species. Our study shows that neryl formate is an aggregation pheromone for *D. farinae* and *D. pteronyssinus*, and has the potential to be used as part of a novel lure-and-kill system for house dust mite control.

KEY WORDS   house dust mite, behavior, aggregation pheromone, neryl formate

Atopic diseases such as asthma, which are associated with inhalation of allergens, are major public health problems in the developed world, with the current economic cost in the United Kingdom estimated to be ~UK£1.05 billion (WHO 2006). The major contributors of house dust mite allergens are present in the fecal pellets (Voorhorst et al. 1964, Tovey et al. 1981, Robinson et al. 1997) of both the American house dust mite, *Dermatophagoides farinae* Hughes, and the European house dust mite, *Dermatophagoides pteronyssinus* Trouessart. Both species are widely distributed throughout the developed world (Fain et al. 1990, Hart 1995, Arlian and Morgan 2003).

A number of approaches have been proposed for reducing the impact of house dust mites upon human health, including physical methods such as impermeable bed covers, or chemicals that act by toxic modes of action, i.e., acaricides. However, because of problems associated with the longevity of known acaricides, the reluctance of asthma patients to use synthetic toxicants in the domestic environment, and general poor exposure of mites to such compounds, other control strategies are required. One approach is to use semiochemicals (naturally occurring behavior- and development-modifying chemicals) to lure dust mites from deep inside furnishings into lure-and-kill trapping systems.

Although the natural product chemistry of house dust mites has been previously studied, the chemical ecology of house dust mites has been insufficiently defined. The chemical profiles of *D. farinae* and *D. pteronyssinus* have been reported (Kuwahara et al. 1990, Kuwahara 1997, 2004), and 2-hydroxy-6-methylbenzaldehyde has been suggested as a sex pheromone for *D. farinae* (Tatami et al. 2001). In addition, aggregation behavior of mites has been reported, but

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the semiochemicals mediating this behavior have not been isolated and identified (Reka et al. 1992, Glass et al. 2001). Therefore, the aim of the current study was to confirm the role and identity of a pheromone responsible for this aggregation behavior, and provide the underpinning science for the development of a novel dust mite lure-and-kill control strategy based on the identified pheromone.

Materials and Methods

House Dust Mites. *D. farinae* were reared as previously described (Skelton et al. 2007). *D. pteronyssinus* were fed on a mixture of ground yeast cells (Allison, London, United Kingdom) and fish flakes (TetraMin, Melle, Germany), at 23–25°C and 75% RH (Spieksma 1967, Arlian et al. 1990), and maintained under these conditions until required for behavioral bioassays and chemical analysis.

Preparation of House Dust Mite Extract and Fractionation. A *D. farinae* extract was prepared using a previously described flotation technique (Fain and Hart 1986), but with hexane used instead of ethanol. *D. farinae* culture (0.1 g, equivalent to 2,500–3,000 mite individuals) was placed separately at the top of glass-measuring cylinders (100 ml) containing saturated NaCl solution (80 ml). After 10–15 min, mites that remained floating were pipetted into a glass vial. Distilled hexane (10 ml) was added and then left at 4°C overnight. The solvent layer of each extract was transferred to a clean vial and dried using anhydrous magnesium sulfate ($MgSO_4$). The extract was filtered and concentrated under a gentle stream of purified nitrogen to 100 μl. A portion of the *D. farinae* extract (50 μl) was fractionated by liquid chromatography over Florisil (60–100 mesh; Sigma-Aldrich, Gillingham, United Kingdom), using distilled hexane (100%), diethyl ether:hexane (5, 10, 20, 50%), diethyl ether (100%), and dichloromethane (100%) as eluants. The collected fractions were gently evaporated under a stream of purified nitrogen, and the solvent was replaced with hexane (50 μl), then stored at -20°C until required for behavioral and chemical studies. A control sample was prepared in a similar manner, but without mites.

Collection of House Dust Mite Volatile Organic Compounds (VOCs). *D. farinae* and *pteronyssinus* mites (0.1 g each) were placed carefully into separate sections of polytetrafluoroethylene (PTFE) tubing (0.25 inches diameter) using a glass pipette tip and gentle suction. Silanized glass wool was inserted into both ends of PTFE tubing, which was then connected at one end via a brass fitting to a glass tube containing TENAX TA (50 mg). A control section of PTFE tubing was also prepared in the same manner, but left empty. At the other end of each section of PTFE tubing, a positive airflow was introduced at 100 ml/min via PTFE tubing (0.125 inches diameter), through a charcoal filter and water, to reduce contamination and provide humidity, respectively. At the same time, a negative airflow was drawn through the TENAX tubes at 100 ml/min. VOC collection was carried out for 24 h

at ambient temperature, upon which the TENAX TA tubes were removed and subjected to either GC or GC-MS analysis. VOC collection was repeated three times for *D. farinae* and twice for *D. pteronyssinus.*

House Dust Mite Behavior. A Y-tube olfactometer was used to observe the behavioral responses elicited by house dust mites to a choice of two odors in a directed airflow (Skelton et al. 2007). For each experiment, a treatment and control stimulus was added (1 μl) to separate filter paper discs (1.5 cm diameter), allowed to dry for 1 min, and placed into each arm of the olfactometer. It was calculated that 20 samples, for each treatment arm, would be required to detect a 40% in effect between treatment at 80% power and 95% significance (STATA 8.2 software). Therefore, experiments were repeated until 20 mites made a choice to either arm for each treatment experiment; the number of mites that failed to make a choice was also recorded. Treatments comprised the following: 1) chromatography fractions derived from the *D. farinae* extract .v. hexane; 2) neryl formate (10 and 100 ng, i.e., amounts equivalent to aggregations of 10–100 mite individuals) .v. hexane; 3) a female *D. farinae* extract .v. neryl formate; 4) a male *D. farinae* extract .v. neryl formate; and 5) a female *D. pteronyssinus* extract .v. neryl formate.

Statistical Analyses. Categorical data from the Y-tube olfactometer bioassays were tested with a χ^2 test for goodness-of-fit, with a Yates correction factor (preference to a particular arm) (Fowler et al. 1998). Time data were log_{10} transformed before parametric data analysis. A three-way analysis of variance (Minitab 11 for Windows) was carried out to analyze whether the log time to make a decision in the treatment arm (neryl formate) was affected by the concentration of neryl formate, gender, and house dust mite species studied.

Gas Chromatography (GC). The *D. farinae* extract and liquid chromatography fractions were analyzed using an Agilent 6890 GC equipped with a poly-dimethylsiloxane capillary column (50 m, 0.32 mm internal diameter (i.d.), 0.52 μm film thickness) fitted with a cool-on-column injector, a deactivated precolumn (1 m, 0.53 mm i.d.), and a flame ionization detector (FID). The GC oven was programmed to hold at 30°C for 0.5 min, then rise to 230°C at 10°C/min, and held at this final temperature for 30 min. The carrier gas was hydrogen. *D. farinae* and *D. pteronyssinus* VOC samples collected on TENAX TA were analyzed using an Agilent 6890 GC equipped with a poly-dimethylsiloxane (HP1) capillary column (50 m, 0.32 mm i.d., 0.52 μm film thickness) and fitted with a programmed temperature vaporization (PTV) unit (Anatune, Cambridge, United Kingdom), which was programmed to rise balistically (20–220°C at 16°/s) to enable thermal desorption of trapped components. The GC oven was programmed to hold at 30°C for 0.5 min, then rise to 120°C at 5°C/min, then rise to 240°C at 10°C/min. The carrier gas was hydrogen.

Coupled GC-Mass Spectrometry (GC-MS). The *D. farinae* extract and liquid chromatography fractions were analyzed using a VG Autospec Ultima mass spec-
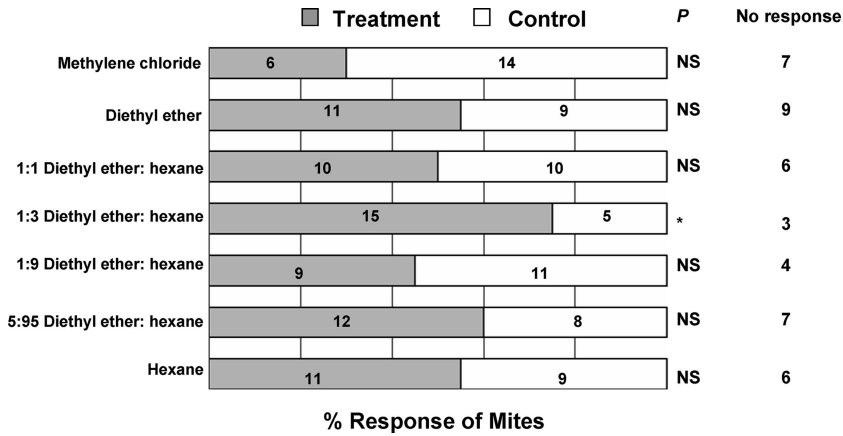
Fig. 1. Behavioral activity of American house dust mites, *D. farinae*, toward liquid chromatography fractions of a hexane extract of *D. farinae* in a Y-tube olfactometer (Skelton et al. 2007). Numbers on bars represent the numbers of responding *D. farinae*. NS = nonsignificant; *, $P < 0.05$. No response = number of mites that did not respond in the apparatus. Categorical data from the Y-tube olfactometer bioassays were tested with a χ^2 test for goodness-of-fit, with a Yates correction factor (preference to a particular arm) (Fowler et al. 1998).

trometer (Fisons Instruments, Manchester, United Kingdom), coupled to an Agilent 6890 GC equipped with a poly-dimethylsiloxane (HP1) capillary column (50 m, 0.32 mm i.d., 0.52 μm film thickness) fitted with a cool-on-column injector and a deactivated precolumn (1 m, 0.53 mm i.d.). *D. farinae* and *D. pteronyssinus* VOC samples collected on TENAX TA were analyzed using Thermo-Finnigan MAT95XP mass spectrometer (Thermo, Bremen, Germany), coupled to a Trace 2000 GC equipped with a poly-dimethylsiloxane (HP1) capillary column (50 m, 0.32 mm i.d., 0.52 μm film thickness) fitted with a PTV unit (Anatune, Cambridge, United Kingdom), which was programmed to rise balistically (20 to 220°C at 16°/s) to enable thermal desorption of trapped components. For both instruments, ionization was by electron impact (70eV, 250°C), and the GC oven was programmed to heat at 30°C for 5 min and then 5°C/min until 250°C. The carrier gas was helium. Tentative identifications of mite-specific peaks were based upon comparison of acquired mass spectra with current MS databases (NIST 2005) or with MS data published in the literature. For liquid extracts and chromatography fractions, tentative identifications were confirmed by peak enhancement on GC columns of different polarity (DB-1 and DB-WAX) with authentic samples (Pickett 1990).

Chemicals. Neryl and geranyl formate were synthesized in one step from commercially available nerol and geraniol, respectively (>97% purity by GC; Sigma-Aldrich, Gillingham, United Kingdom) using formic acid and 1,3-dicyclohexylcarbodiimide. Structures were confirmed by comparison of ^{1}H and ^{13}C nuclear magnetic resonance and MS data with literature values. Hexane, diethyl ether, and dichloromethane (methylene chloride) were distilled before use in behavioral and chemical studies.

Quantification of Neryl Formate Per House Dust Mite. Samples of *D. farinae* and *D. pteronyssinus* were transferred from culture cells to petri dishes and sexed using a diagnostic key and diagrams (Fain et al. 1990, Colloff and Spieksma 1992), and the desired amount (25, 50, or 100) of either males, females, or mixed adults was transferred into a vial containing distilled hexane (1.5 ml) using a single paintbrush hair. Samples were left at 4°C overnight, then concentrated to a volume of 50 μl under a gentle stream of nitrogen, before GC analysis. The amounts of neryl formate in extracts were quantified using a single point external standard quantification method, i.e., using peak area data from acquired GCs, and comparing with the peak area of a known amount of neryl formate.

Results

House Dust Mite Behavior Response to Extracts and Chromatography Fractions. In Y-tube olfactometer studies measuring the response of male and female *D. farinae* to extracts and chromatography fractions, a significant attractive response was only observed to the chromatography fraction collected with 20% diethyl ether:hexane ($x^2 = 4.05$, df = 1, $P < 0.05$, $n = 20$) (Fig. 1). No significant difference was observed for the unfractionated extract and the other chromatography fractions, compared with solvent controls.

House Dust Mite Chemical Analysis. GC and coupled GC-MS analysis of a hexane extract of *D. farinae* revealed the presence of several components that were not present in a control extract (Fig. 2). There was no discernable difference in the range of components present in male and female samples (data not shown). Major components that were tentatively identified included 2-hydroxy-6-methylbenzaldehyde, neral, geranial, neryl or geranyl formate, undecane, tetradecane, and pentadecane and heptadecane. GC and GC-MS analysis of the liquid chromatography fractions revealed the presence of a peak uniquely present in the behaviorally active 20% diethyl ether:hexane chromatography fraction, which was tenta-
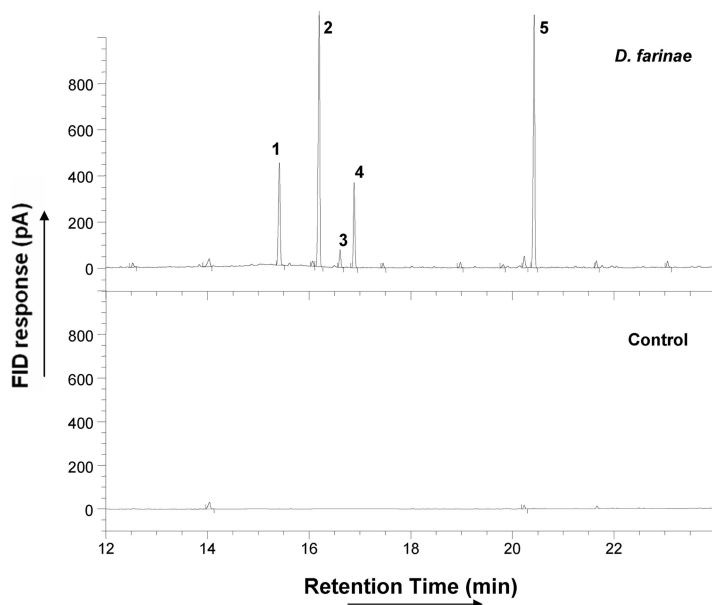
Fig. 2. GC-FID analysis of a hexane extract of American house dust mites, *D. farinae,* and a hexane control, using a 50 m × 0.32 mm i.d. DB-1 column. Peak numbers: 1 = 2-hydroxy-6-methylbenzaldehyde; 2 = neral; 3 = geranial; 4 = neryl formate; 5 = pentadecane.

tively identified as either neryl or geranyl formate. Identification of this peak as the neryl isomer was confirmed by the peak enhancement of the peak in the active fraction with an authentic sample of neryl formate, synthesized from nerol, on two GC columns of differing polarity. Using a single point external standard quantification method, the amount of neryl formate per *D. farinae* mite was calculated to be 1.32 ± 0.2 ng and 3.3 ± 0.3 ng for males and females, respectively. The amount of neryl formate per *D. pteronyssinus* mite was 0.5 ± 0.01 ng and 1.13 ± 0.11 ng for males and females, respectively.

Analysis of the other fractions revealed that 2-hydroxy-6-methylbenzaldehyde was found in the behaviorally inactive 50 and 100% diethyl ether fractions (data not shown).

VOC Collection and Analysis. GC-MS analysis of *D. farinae* and *D. pteronissinus* VOCs collected for 24 h on TENAX TA tubes (50 mg) revealed the presence of compounds that previously were isolated in the *D. farinae* hexane extract (Fig. 3). For *D. farinae,* VOCs included 2-hydroxy-6-methylbenzaldehyde, neral, geranial, neryl formate, and tridecane. For *D. pteronyssinus,* geranial and neryl formate were also detected in
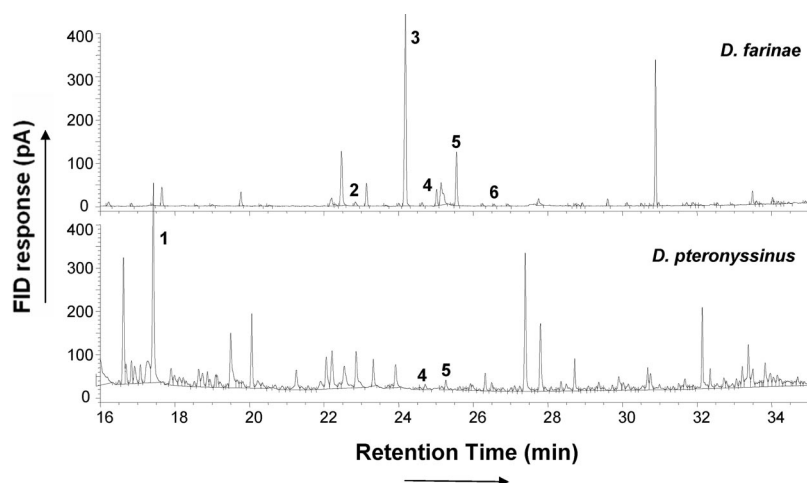

Fig. 3. GC-FID analysis of VOCs emitted by American house dust mites, *D. farinae,* and European house dust mites, *D. pteronyssinus,* using a 50 m × 0.32 mm i.d. DB-1 column. Peak numbers: 1 = limonene; 2 = 2-hydroxy-6-methylbenzaldehyde; 3 = neral; 4 = geranial; 5 = neryl formate; 6 = tridecane.
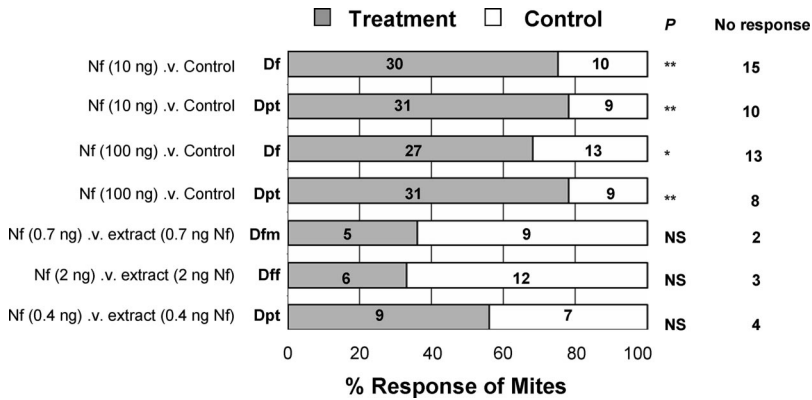
Fig. 4. Behavioral activity of the American house dust mite, *D. farinae* (Df), and the European house dust mite, *D. pteronyssinus* (Dpt), in response to neryl formate (Nf), either tested versus a solvent control or a conspecific extract containing neryl formate at an identical level (extract; Dfm = Df males, Dff = Df females), using a Y-tube olfactometer (Skelton et al. 2007). Numbers on bars represent the numbers of responding *D. farinae*. NS = nonsignificant; *, $P < 0.05$; **, $P < 0.01$. No response = number of mites that did not respond in the apparatus. Categorical data from the Y-tube olfactometer bioassays were tested with a χ^2 test for goodness-of-fit, with a Yates correction factor (preference to a particular arm) (Fowler et al. 1998).

the VOCs. Compounds specific to *D. pteronyssinus* included limonene, and heptadecadiene and heptadecene isomers.

House Dust Mite Behavior Response to Neryl Formate. Male and female *D. farinae* were significantly attracted to neryl formate at a dose of 10 ng ($x^2 = 9.0$, df = 1, $P < 0.01$, $n = 40$) and 100 ng ($x^2 = 4.2$, df = 1, $P < 0.05$, $n = 40$) (Fig. 3). Male and female *D. pteronyssinus* were also significantly attracted to neryl formate at 10 ng ($x^2 = 11.0$, df = 1, $P < 0.01$, $n = 40$) and 100 ng doses ($x^2 = 11.0$, df = 1, $P < 0.01$, $n = 40$) (Fig. 4). However, variations in the responses between genders were observed. Male *D. farinae* only demonstrated a significant response to neryl formate at 100 ng ($x^2 = 6.1$, df = 1, $P < 0.05$, $n = 20$), whereas females demonstrated a significant response only at 10 ng ($x^2 = 6.1$, df = 1, $P < 0.05$, $n = 20$). Male *D. pteronyssinus* demonstrated a significant response to neryl formate at 10 ng only ($x^2 = 6.05$, df = 1, $P < 0.05$, $n = 20$), whereas female *D. pteronyssinus* demonstrated a significant response to both 10 ng ($x^2 = 4.1$, df = 1, $P < 0.05$, $n = 20$) and 100 ng ($x^2 = 8.4$, df = 1, $P < 0.01$, $n = 20$). There were no significant differences in the log time taken by *D. farinae* and *D. pteronyssinus* to move past the 1-cm mark point along the arm containing neryl formate when analyzing concentration (10 ng against 100 ng of neryl formate) ($F = 2.4$; df = 1, 1; $P = 0.125$) or gender effects ($F = 1.6$; df = 1, 1; $P = 0.208$). However, a significant difference was found between the log time taken by *D. farinae*, compared with *D. pteronyssinus*, to move past the 1-cm mark point of the arm containing neryl formate ($F = 10.3$; df = 1, 1; $P < 0.02$).

House Dust Mite Behavior Response to Synthetic Neryl Formate Versus Neryl Formate in Conspecific Extracts. For *D. farinae* males, there was no significant difference in the response to synthetic neryl formate (0.7 ng) or a male *D. farinae* extract containing neryl formate at the same level ($x^2 = 0.10$, df = 1, $P = 0.75$,

$n = 14$). Similarly, for *D. farinae* females, there was also no significant difference in the response to either neryl formate (2 ng) or a female *D. farinae* extract containing neryl formate at the same level ($x^2 = 1.38$, df = 1, $P = 0.24$, $n = 18$) (Fig. 4). Likewise, the responses of *D. pteronyssinus* females to either synthetic neryl formate (0.4 ng) or a female *D. pteronyssinus* extract containing neryl formate at the same level were not significantly different ($x^2 = 0.06$, df = 1, $P = 0.80$, $n = 16$) (Fig. 4).

Discussion

All of the compounds identified from *D. farinae* and *D. pteronyssinus* in the current study have been previously reported. Thus, the flotation technique (Fain and Hart 1986) is suitable for VOC extraction from house dust mites. To our knowledge, this was the first example of dynamic headspace collection being used in the collection of VOCs emitted by house dust mites.

Before our study, neryl formate had only been tentatively identified as a component of *D. farinae* and *D. pteronyssinus* extracts (Kuwahara et al. 1990, Tatami et al. 2001). Our results show unequivocally that *D. farinae* and *D. pteronyssinus* produce and emit neryl formate, and, for the first time, show that this VOC elicits significant directional responses by male and female *D. farinae* and *D. pteronyssinus* at low levels (10 and 100 ng) that mimic mite aggregations. Other astigmatic mite aggregation pheromones possess biological activity at these levels, e.g., *Caloglyphus polyphyllae* Zakhvatkin mites significantly respond to β-acaridial at a level of 10 ng (Shimizu et al. 2001), and *Lardoglyphus konoi* Sasa & Asanuma significantly responds to (1*R*,3*R*,5*R*,7*R*)-lardolure at 10 ppm (10 ng/μl) (Kuwahara et al. 1991). In our study, when synthetic neryl formate was tested against neryl formate at the same concentration in a house dust mite extract, neither *D. farinae* nor *D. pteronyssinus* showed a significant pref-

erence for either arm. These results strongly indicate that neryl formate accounts entirely for the activity previously demonstrated for the behaviorally active chromatography fraction. There was a significant difference in the time taken by mites to move along the arm containing neryl formate when using either *D. farinae* or *D. pteronyssinus,* but this does not necessarily imply that *D. pteronyssinus* walk faster than *D. farinae,* as the track the mite moved along was not recorded.

Previous reports in the literature suggested that neryl formate acts as an alarm pheromone for other astigmatic mite species, including the storage mite, *Tyrophagus putrescentiae* Schrank (Kuwahara et al. 1975); the bulb mite, *Rhizoglyphus robini* Claparede (Kuwahara et al. 1988; *Rhizoglyphus setosus* Manson (Akiyama et al. 1997); and *Histiogaster rotundus* Woodring (Hiraoka et al. 2003). It has also been reported that 2-hydroxy-6-methylbenzaldehyde (also detected in our study) is a sex pheromone for *D. farinae* (Tatami et al. 2001), *Acarus immobilis* Griffiths (Sato et al. 1993), *Aleuroglyphus ovatus* Troupeau (Kuwahara et al. 1992), and *Cosmoglyphus hughesi* Samsinak (Ryono et al. 2001). In our study, a Y-tube olfactometer designed specifically for use with house dust mites (Skelton et al. 2007) was used to assess the response to airborne semiochemicals acting at a distance. Thus, the attraction of *D. farinae* and *D. pteronyssinus* to neryl formate, when tested at a physiologically relevant level, represents a surprising, but important discovery, and implies an important role for this compound as an airborne aggregation pheromone. The lack of attraction of *D. farinae* and *D. pteronyssinus* to extracts containing 2-hydroxy-6-methylbenzaldehyde reinforces the hypothesis that this is a close contact sex pheromone that initiates mounting behavior in male *D. farinae* (Tatami et al. 2001).

The benefit of an aggregation pheromone to house dust mites remains unclear. *D. farinae* have been observed clustering in the laboratory (Reka et al. 1992), in particular, males at hydrating conditions (75% RH), suggesting semiochemicals may be involved (Glass et al. 1998). Both *D. farinae* and *D. pteronyssinus* have been observed clustering together by one of the authors (A.C.S.) in the current study. It has been suggested that the clustering behavior is initiated by an arrestant-aggregation pheromone in house dust mite feces, but the study observing this behavior did not identify the chemical or chemicals involved (Reka et al. 1992). Therefore, the behavioral responses observed to neryl formate suggest that it may be involved in the clustering behavior of house dust mites. The ecological benefits of clustering could be to either protect themselves from dehydration or provide a defense mechanism. Mites may cluster together to reduce the surface area that is exposed to the drier environment and subsequently prevent dehydration. There is evidence to suggest that arthropods aggregate to form a superorganism to reduce water loss by reducing the surface area of the individual arthropod, and this behavior has been observed in *Stenotarus rotundus,* the tropical fungus beetle (Yoder et al.

1992). Clustering together may serve as a defense mechanism to protect against potential predators, e.g., by the Cheyletidae mites (Colloff 1991), and the formation of big clusters of house dust mites in the homes may disorientate the predators at locating an individual mite (Franz et al. 2001). Alternatively, as neryl formate is a chemical commonly found in astigmatic mites (Kuwahara 2004), it may be involved in house dust mite recognition of a population presence with subsequent species-specific semiochemical cues used later to locate a mate, e.g., 2-hydroxy-6-methylbenzaldehyde, which initiates mounting behavior in male *D. farinae* (Tatami et al. 2001), and is likely to be a close contact pheromone.

The lack of behavioral studies using house dust mites, especially those conducted in domestic households, has contributed to the problem of house dust mite control. For neryl formate to act as an effective pheromone lure in a control product, the distance and the length of time that *D. farinae* and *D. pteronyssinus* can locate the lure need to be determined. Further studies, including semifield testing, are underway to investigate these parameters. Neryl formate is a cheap, readily available natural product and is a permitted food additive. Therefore, it has the potential to be developed as part of a lure-and-kill system for controlling house dust mite populations, and ultimately help in alleviating the symptoms associated with atopic diseases.

Acknowledgments

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