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# A male sex pheromone in a parasitic wasp and control of the behavioral response by the female's mating status

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## Summary

**Male insects may increase their chance of successful reproduction by releasing pheromones that attract females or elicit sexual acceptance. In parasitic wasps, male pheromones have been suggested for a few species but no chemicals have been identified so far. Here we report the first identification of a male sex pheromone in parasitic Hymenoptera. In abdomens of male jewel wasps, *Nasonia vitripennis* Walker, we found a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL), which was released intermittently and attracted virgin females, but no males, in an olfactometer bioassay. However, only a few minutes after copulation mated females avoided the male-derived pheromone. Neither preference nor avoidance was shown by mated females after 24 h and even after they had been allowed to oviposit for 6 days. *Nasonia vitripennis* females normally mate only once. Thus, their variable response to the sex attractant depending on the mating status makes sense from an evolutionary perspective. Firstly, it increases the chance of virgins to be inseminated. Secondly, by terminating the response or even avoiding the male pheromone, mated females decrease the probability of encountering males and being disturbed by their courtship activities when searching for new oviposition sites.**

Key words: 5-hydroxy-4-decanolide, Hymenoptera, mating behavior, *Nasonia vitripennis*, parasitic wasp, parasitoid, Pteromalidae, sex pheromone.

## Introduction

Parasitic wasps are carnivorous insects that develop by feeding in or on the body of other insects. They play a crucial role in natural and agricultural ecosystems by maintaining species diversity and controlling insect pests (Quicke, 1997). The reproductive success of parasitoid males depends on their ability to locate and inseminate receptive females. As in most other insects, sex pheromones are supposed to be of substantial importance during the mate finding process of parasitic wasps (Godfray, 1994). However, only a few sex pheromones have been identified so far (reviewed by Kainoh, 1999; Ayasse et al., 2001; Keeling et al., 2004). These are exclusively female-derived compounds which are either of high volatility and attract males over long distances (Eller et al., 1984; Swedenborg and Jones, 1992; Swedenborg et al., 1994) or of low volatility and mediate arrestment and male courtship behavior at close range (Shu and Jones, 1993; Syvertsen et al., 1995; Sullivan, 2002; Steiner et al., 2005; Steiner et al., 2006). Nothing is known about the chemistry of male-derived sex pheromones in parasitic wasps, although a few behavioral studies suggest that they exist (van den Assem et al., 1980; Gonzalez et al., 1985).

The physiological state of insects can have drastic impacts on their reproductive behavior. When ejaculating, males not only transfer sperm but also bioactive molecules, from their accessory glands, that induce a variety of physiological and behavioral changes in females. Numerous studies [see Gillott (Gillott, 2003) and references therein] have shown that these mostly proteinaceous compounds may not only induce refractoriness (rejection of courting males) but also arrest biosynthesis and release of female sex pheromones. By this means, males that gained the first copulation may increase their chance of siring a maximum number of the female's offspring. Studies on the Mediterranean fruit fly *Ceratitis capitata* provided the first evidence that the female mating status may also influence the responsiveness to olfactory stimuli (Jang, 1995; Jang, 2002). After mating or experimental injection of accessory gland fluid, the female preference switched from the male sex pheromone to host odors. This behavioral switch of the females did not occur until 24–48 h after treatment with the accessory gland fluid (Jang, 1995).

The jewel wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae) is a gregarious parasitoid that attacks pupae of several cyclorraphous fly species. It is one of the most

investigated parasitoid species (Quicke, 1997) and, thus, can be considered as a model organism for the study of parasitic wasp biology. Both male and female sex pheromones have been reported in *N. vitripennis*. Males respond to female cuticular hydrocarbons by courtship behavior (Steiner et al., 2006) and release a still unknown aphrodisiac from their mandibular gland that triggers the readiness to copulate in the female (van den Assem et al., 1980). Furthermore, males reportedly mark territories chemically after copulation. These marks are attractive to females and other males (van den Assem, 1986). In the course of our recent study on the female courtship pheromone of *N. vitripennis* (Steiner et al., 2006) we found two chemicals in whole body extracts from older males that were absent in those from females (J.R., unpublished). Here we report the identification of these compounds and demonstrate that they function as a male sex pheromone, attracting females. Apart from structure elucidation, we investigated in which part of the insect the pheromone is located and whether the pheromone titer depends on the age of the male parasitoids. Furthermore, we determined release dynamics of pheromone from individual males and studied whether the mating status of females influences their responsiveness to the male signal.

## Materials and methods

### *Insects*

*Nasonia vitripennis* Walker wasps were collected from a bird's nest near Hamburg, Northern Germany. Laboratory cultures were kept at 25°C and 60% relative humidity with a daily light:dark cycle of 16 h:8 h. Thirty to forty freshly emerged *N. vitripennis* of both sexes were placed in Petri dishes with 50 freeze-killed puparia of the green bottle fly *Lucilia caesar* and kept there until their death. The next generation emerged from the host puparia after a development time of 14–15 days. Parasitoid pupae were removed from host puparia 1–2 days prior to eclosion and kept individually in 1.5 ml microcentrifuge tubes until emergence. Mated females were obtained by keeping one virgin female together with a male for 5 min in an observation chamber (0.5 cm high, 7 cm diameter). Each mating was observed under a stereo microscope. Subsequently, females were kept individually in microcentrifuge tubes for either 5 min or 24 h until used in the bioassays. These two periods were selected to study whether the mating status influences the female behavioral response right after (5 min) and/or later (24 h) following copulation. A third group of mated females was allowed to parasitize fresh host pupae for 6 days before the bioassay.

### *Pheromone extraction and fractionation*

Virgin males of different ages [<1 h ($N$=26), 1 day ($N$=19), 2 days ($N$=17), 3 days ($N$=15)] and 1-day-old virgin females ($N$=15) were killed by freezing, transferred to a 1 ml glass vial equipped with a 100 μl micro insert, and extracted singly for 30 min with 5 μl of dichloromethane containing 50 ng $\mu l^{-1}$ methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) as an internal standard. To identify the site of pheromone storage, 3-day-old unmated males were dissected with a scalpel into head, thorax and abdomen, and subsequently tagmata were extracted separately as described above ($N$=5 for each segment). Aliquots (1 μl) were analyzed by coupled gas chromatography–mass spectrometry (GC–MS). Amounts of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL) in individuals were estimated by relating peak areas to the internal standard. For testing bioactivity of natural (4*R*,5*R*)- and (4*R*,5*S*)-HDL, pheromone extracts were fractionated by adsorption chromatography. Groups ($N$=20) of 3-day-old males were killed by freezing and extracted for 30 min with 100 μl of dichloromethane. After removal of the supernatant, the cadavers were washed twice with 100 μl of dichloromethane. The combined extracts were applied to a silica gel cartridge (25 mg; International Sorbent Technology, Glamorgan, UK) and rinsed twice with 250 μl of dichloromethane. The diastereomers of HDL were eluted with 250 μl of methanol. After removal of the methanol under a gentle stream of nitrogen the residue was reconstituted in 100 μl of dichloromethane for bioassay.

### *Volatile collection*

The volatile collection system consisted of a mini vacuum pump (Neolab, Heidelberg, Germany) that was connected by a piece of Teflon tube to an adsorption filter (65 mm length, 35 mm diameter; Gränicher and Quartero, Daumazan, France) designed for closed loop stripping analysis (CLSA) (Grob and Zürcher, 1976) and equipped with a 1-mm charcoal layer (5 mg) for volatile adsorption. Single insects (3-day-old males, $N$=12) were directly released into the filter tube and the open end of the tube was connected to another glass tube (75 mm length, 36 mm diameter) containing 150 mg of charcoal to clean the air stream before entering the CLSA-tube. An air stream of 40 ml $min^{-1}$ was sucked through the tube system for 5 h. Adsorbed volatiles were eluted with 25 μl of dichloromethane containing 5 ng $\mu l^{-1}$ methyl undecanoate as an internal standard and used for chemical analysis by GC–MS. Amounts of HDL-diastereomers per sample were quantified by relating peak areas to the internal standard. To test whether HDL was released by the males continuously or intermittently, we repeated the experiment ($N$=10) but exchanged the adsorption tube every hour (referred to as 5×1 h). Thereby, it was possible to estimate HDL amounts released by individual males for each hour separately. Volatile sampling of *N. vitripennis* males was performed during the photophase (10:00 h and 18:00 h), no food or water was offered to the insects to avoid the danger of contaminations.

### *GC–MS analysis*

Extracts were subjected to GC–MS analysis on a Fisons 8060 gas chromatograph (Fisons Instruments, Mainz-Kastel, Germany) equipped with a DB-5ms capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) operated in splitless mode (injector temperature: 240°C) and coupled to a Fisons MD800 quadrupole MS running in the electron impact (EI) mode at 70 eV. Helium was used as the carrier gas at a head pressure of 5 kPa (flow rate

1.0 ml $min^{-1}$). Initial oven temperature was 80°C, increased at 5°C $min^{-1}$ to 280°C and held for 30 min. Linear retention indices (RI) of the male-specific double peak in natural extracts were estimated by co-injection of a mixture of n-alkanes with chain lengths between seven and 30 carbon units (Sigma-Aldrich, Steinheim, Germany) (van den Dool and Kratz, 1963). The compounds eluting at RI=1592 and 1608 were identified as a pair of diastereomers of HDL by comparison of mass spectra and RI with those of authentic reference chemicals which were synthesized as described elsewhere (Garbe and Tressl, 2003). The two threo-enantiomers, (4*R*,5*R*)- and (4*S*,5*S*)-HDL, eluted before the erythro-enantiomers (4*R*,5*S*)- and (4*S*,5*R*)-HDL. Threo- and erythro-enantiomers of HDL were separated on a chiral β-DEX 225 GC column (=25% 2,3-di-*O*-acetyl-6-*O*-TBDMS-β-cyclodextrin in polydimethylsiloxane, 30 m× 0.25 mm diameter, 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). Hydrogen was used as carrier gas. The flow was adjusted to 25 cm $s^{-1}$ at an oven temperature of 120°C. The GC was operated in splitless mode (15 s) at an injector temperature of 240°C. Initial oven temperature was 80°C, increased at 3°C $min^{-1}$ to 220°C and held for 10 min. All four stereoisomers were separated with the two threo-enantiomers eluting before the erythro-enantiomers. Threo-enantiomers were only partially resolved with (4*R*,5*R*)-HDL eluting before (4*S*,5*S*)-HDL whereas the erythro-enantiomers were totally resolved with (4*R*,5*S*)-HDL eluting before (4*S*,5*R*)-HDL. The absolute configuration of the natural products was finally established to be (4*R*,5*R*)- and (4*R*,5*S*)-HDL by co-injection of the polar fraction from male extracts with authentic reference compounds.

### *Bioassays*

The experiments were carried out in a four-chamber static-air-flow olfactometer at 20±1°C under illumination of a microscope lamp. The olfactometer consisted of an acrylic cylinder (1.5 cm high, 7 cm diameter) divided into four chambers by crosswise arranged vertical plates. The top of the cylinder was covered by plastic gauze (mesh 0.1 mm) functioning as a walking arena for the parasitoids. A lid consisting of a plastic ring (4 mm high, 7 cm diameter) and a second gauze (mesh 0.1 mm) was placed on top of the cylinder to prevent the parasitoids from escaping. Quarters of MN 615 filter paper discs (5.5 cm diameter; Macherey and Nagel, Düren, Germany) were placed in each of the four chambers underneath the walking arena. The following samples were tested: 5 μl of fractions containing natural HDL diastereomers (representing one male equivalent, see above; experiment 1) or a synthetic mixture of (4*R*,5*R*)- and (4*R*,5*S*)-HDL (ratio 1:1.3) in dichloromethane at doses of 80 or 160 ng HDL (experiment 2). To determine whether the female response is enantioselective, we also tested 80 ng of either the naturally occurring (4*R*,5*R*)- or the (4*S*,5*S*)-enantiomer that does not occur in the insects (see Results, experiment 3). Samples were applied to the filter paper of one chamber (test) and equal amounts of the clean solvent were applied to the filter papers of the remaining chambers. The opposite chamber was considered as control chamber whereas the remaining two chambers adjacent to the test chamber were considered as buffer zones. Parasitic wasps (virgin females, mated females 5 min or 24 h after copulation, mated females 6 days after copulation with oviposition opportunity, and unmated males, *N*=20 for each type) were released singly into the walking arena using a fine paintbrush. The time spent by wasps in the sectors above the four chambers was recorded for 300 s using the The Observer 3.0 computer software (Noldus, Wageningen, The Netherlands). To avoid biased results caused by possible side preferences of the parasitoids, samples were assigned randomly to one of the chambers and the olfactometer was rotated by 90° after every wasp.

### *Statistical analysis*

Amounts and ratios of (4*R*,5*R*)- and (4*R*,5*S*)-HDL in extracts from males of different age were analyzed by a one-way analysis of variance (ANOVA) followed by least significant difference test (LSD) for individual comparisons. Amounts of HDL released by individual males within 5 h were compared to the summed amounts released within 5×1 h by a Mann–Whitney *U*-test. Residence time of males spent above the test and control chamber in the bioassays were compared by a *t*-test for dependent samples. Statistical analyses were done using Statistica 4.5 scientific software (StatSoft, Hamburg, Germany).

## Results

Chemical analysis of solvent extracts of *N. vitripennis* males and females by GC–MS revealed a male-specific double peak

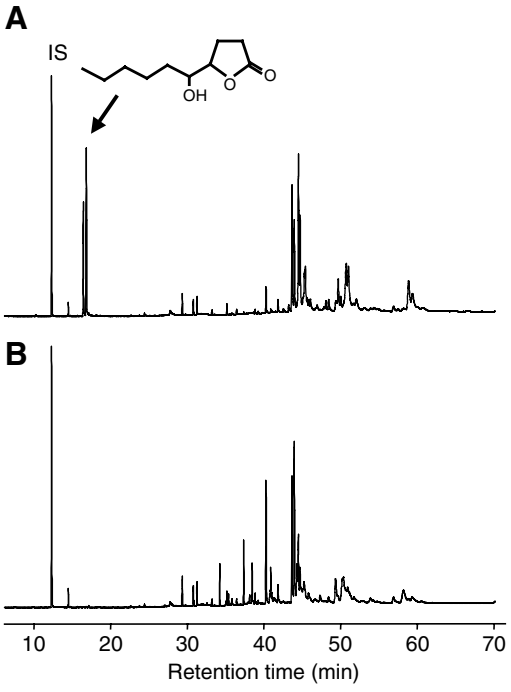


Fig. 1. Total ion current chromatograms of dichloromethane extracts from (A) male and (B) female *N. vitripennis* using a non-polar DB-5 ms capillary column. The arrow indicates the male-specific double peak of diastereomers of 5-hydroxy-4-decanolide. IS, internal standard (50 ng methyl undecanoate).

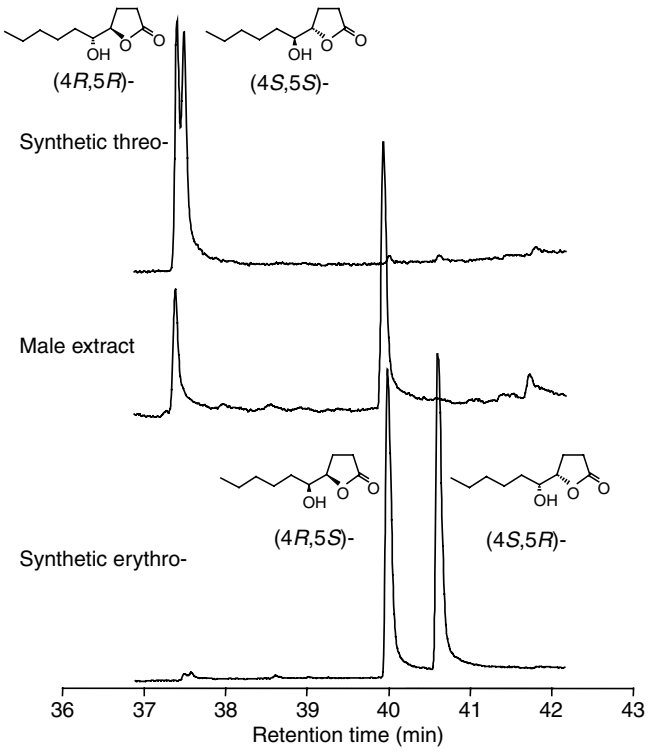


Fig. 2. Separation of synthetic threo- and erythro-enantiomers of 5-hydroxy-4-decanolide (HDL) on a chiral β-DEX 225 GC column. Extracts from *N. vitripennis* males contained (4*R*,5*R*)- and (4*R*,5*S*)-HDL.

with almost identical mass spectra. MS data and retention indices matched those of synthetic threo- and erythro-diastereomers of HDL (Fig. 1) (Garbe and Tressl, 2003).

Enantiomeric pairs of threo- and erythro-HDL were separated on a chiral GC column; males produced (4*R*,5*R*)- and (4*R*,5*S*)-HDL, respectively (Fig. 2). Amounts of HDL were close to zero in freshly emerged males, increased within the first 2 days after emergence and remained at a constant level on day 3 (Fig. 3). The ratio of (4*R*,5*S*)-/(4*R*,5*R*)-HDL changed successively with increasing age of the males in favor of (4*R*,5*S*)-HDL ($F$=9.980, d.f.=3, $P$<0.001, one-way ANOVA). Separate extraction of different body segments revealed that 99±0.8% (mean ± s.e.m.) of HDL was present in the abdomen and only trace amounts were detectable in extracts of head and thorax, suggesting that the abdomen is the site of biosynthesis. After sampling of volatiles from individual males (5 h), total HDL-diastereomers were found to be 80±27 ng per male (mean ± s.e.m.). Total amounts released by individual males within 5 h were not significantly different from summed amounts released in the 5×1 h experiment (90.9±20.4 ng per male, $P$=0.391, Mann-Whitney $U$-test). 80% of the males were found to release HDL intermittently, as shown by clearly increased amounts within single hours of the sampling period and decreased release rates in between (Fig. 4). Therefore, the bouts of pheromone release were highly variable among individual males.

Virgin females, but not males, were strongly attracted by the natural HDL-diastereomers (1 male equivalent of a whole body extract cleaned up by adsorption chromatography; experiment 1, Fig. 5A), as well as by 80 and 160 ng of the synthetic mixture of (4*R*,5*R*)- and (4*R*,5*S*)-HDL (experiment 2, Fig. 5B). The response of virgin females was not enantioselective because both the naturally occurring (4*R*,5*R*)-enantiomer, as well as the

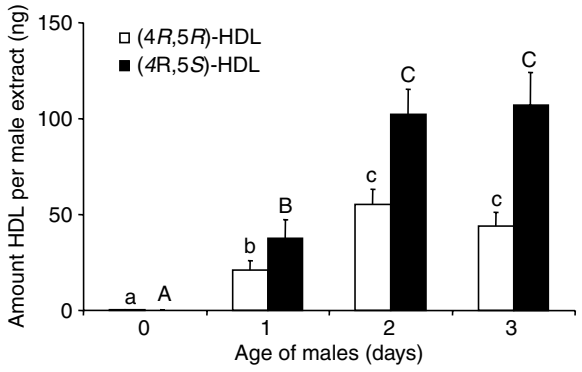


Fig. 3. Amounts (mean ± s.e.m.) of 5-hydroxy-4-decanolide in whole body extracts from *N. vitripennis* males of various age (different lower- and uppercase letters indicate significant differences for each diastereomer at $P$<0.05, one-way ANOVA and LSD test).

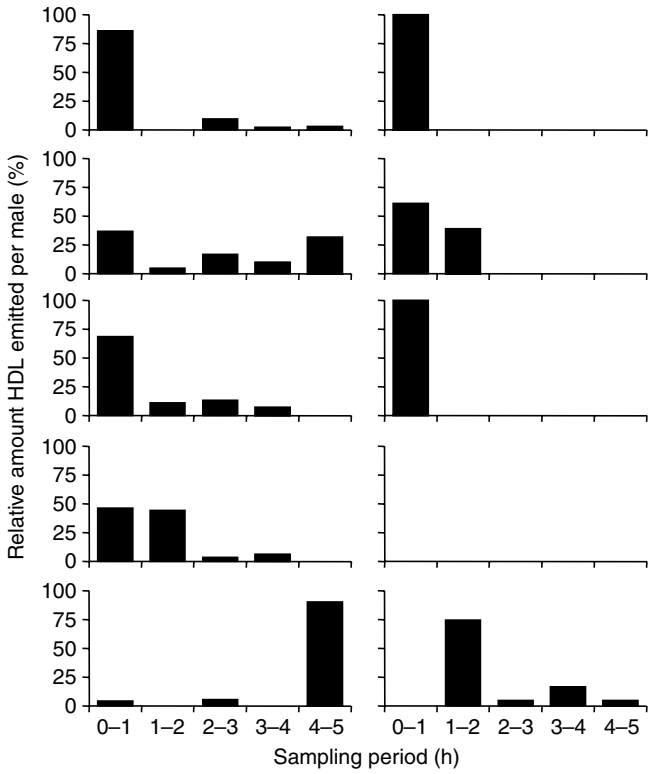


Fig. 4. Release dynamics of 5-hydroxy-4-decanolide (total amounts) by *N. vitripennis* males during a 5-h sampling period ($N$=10). Each bar diagram represents the results from one individual male. Amounts released per hour are presented as the percentage of the total amount released in 5 h. Volatile sampling was performed during the photophase between 10:00 h and 18:00 h.

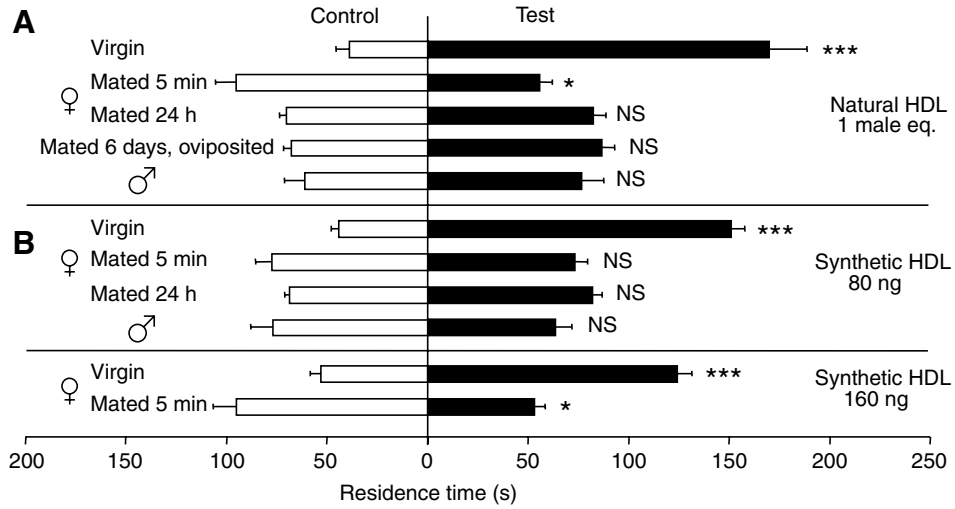


Fig. 5. Residence time (mean ± s.e.m.) of *N. vitripennis* females and males in odor and control fields of a two-choice olfactometer. Females were either virgin or mated and tested 5 min, 24 h or 6 days after copulation. The 6-day ones had the opportunity to oviposit. Tested males had no copulation experience. Odor fields were treated with (A) one male equivalent (eq.) of natural 5-hydroxy-4-decanolide (HDL; experiment 1), or (B) synthetic (4*R*,5*R*)- and (4*R*,5*S*)-HDL (ratio 1:1.3) at doses of 80 or 160 ng (experiment 2). Control fields were treated with the pure solvent. Asterisks indicate significant differences (**P*<0.05, ****P*<0.001); NS, not significant (*t*-test for dependent samples, *N*=20).

(4*S*,5*S*)-enantiomer that does not occur in the parasitoids, were attractive at a dose of 80 ng (experiment 3, Fig. 6).

Five minutes after copulation females avoided one male equivalent of natural HDL or 160 ng of the synthetic mixture (Fig. 5A,B). Neither preference nor avoidance of all samples offered was shown by mated females after 24 h. Females that had been allowed to oviposit for 6 days, were still neither attracted nor repelled by natural HDL, but there was a tendency to prefer the test field ($P$=0.06).

## Discussion

Mating systems of insects are often characterized by strong male–male competition for receptive females (Choe and Crespi, 1997). One mechanism in male insects to increase the chance of successful reproduction is the release of sex pheromones that attract females or elicit sexual acceptance (Wyatt, 2003). However, in parasitic wasps the use of female sex pheromones is thought to be more common (Godfray, 1994) and involvement of male-derived pheromones has only rarely been demonstrated (van den Assem et al., 1980; Gonzalez et al., 1985). The present study reports the first identification of a male sex pheromone in parasitic Hymenoptera. Males of *N. vitripennis* synthesize HDL-diastereomers in their abdomen and release these chemicals that attract virgin females, but not males. *Nasonia vitripennis* is a gregarious parasitoid developing in groups of 10–20 individuals within a single host. The protandrous males often wait at the emergence hole of the puparium they emerged from to mate with their sisters. Thereby, dominant males may aggressively chase away inferior competitors (van den Assem, 1986). By releasing the sex pheromone, males may signal genetic quality and influence the outcome of this contest. In fact, males may effectively 'call' for females, as indicated by the intermittent release of the pheromone even in the absence of females. The fact that HDL is biosynthesized in the male abdomen, suggests that it is not identical with the postulated aphrodisiac that is released from the mandibular gland of *N. vitripennis* males during courtship and triggers the readiness to mate in females (van den Assem et al., 1980). This conclusion is supported by the result that males released HDL even when their mouthparts had been sealed with quick-drying superglue

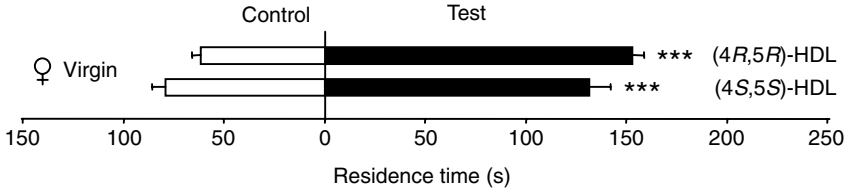


Fig. 6. Mean residence time of virgin females in test and control fields of a two-choice olfactometer. Test fields were treated with (4*R*,5*R*)- or (4*S*,5*S*)-5-hydroxy-4-decanolide (HDL) at doses of 80 ng (experiment 3). Control fields were treated with the pure solvent. ***Significant differences at $P$<0.001 (*t*-test for dependent samples, *N*=20).

(L.M.S., unpublished). Thus, further components of the *N. vitripennis* chemical communication system remain to be discovered.

The present study clearly demonstrates that the responsiveness of females to the male sex attractant is affected by their mating status. Copulation of *N. vitripennis* females immediately switched off their attraction to HDL, and shortly after copulation they even avoided the chemical signal that indicates the presence of males. A similar mechanism was shown before for the Mediterranean fruit fly *Ceratitis capitata*: after copulation, the female behavioral preference switched from the male sex pheromone to host fruit odors (Jang, 1995; Jang, 2002). However, in *C. capitata* females did not switch their behavior until 24–48 h after mating (Jang, 1995) and it was not determined whether this switch was due to avoidance of the male pheromone or an increased attractiveness of the host fruit odor. Nevertheless, the observed phenomenon was clearly shown to be associated with accessory gland fluids transferred by males to the females during copulation. This might also be the case in *N. vitripennis,* although it has been shown that refractoriness in this species is initiated during post-copulatory courtship behavior. Van den Assem and Visser (van den Assem and Visser, 1976) reported that *N. vitripennis* females can be provoked to remate by experimentally preventing male post-copulatory behavior. However, we found the described behavioral switch concerning HDL also in those females that had copulated but did not experience post-copulatory courtship (J.R., unpublished). This strengthens the conclusion that it is the copulation itself rather than post-copulatory courtship that triggers the observed behavioral switch of *N. vitripennis* females. Under normal circumstances, *N. vitripennis* females mate only once (van den Assem, 1986) and therefore, their variable response depending on the mating status makes sense from an evolutionary perspective. Firstly, it increases the chance of virgins to be inseminated. Secondly, by terminating the response to HDL or even avoiding the male pheromone, mated females decrease the probability of encountering males and being disturbed by their courtship activities when searching for new oviposition sites. Males in return will be less likely to engage in unavailing courtship activities towards non-receptive females.

To the best of our knowledge this is the first report of (4*R*,5*R*)- and (4*R*,5*S*)-HDL in insects, although long-chain lactones, including decanolides, are common insect semiochemicals (e.g. Howard et al., 1983; Nishida et al., 1996; Larsson et al., 2003). The response of insects to chiral lactones can be extremely enantioselective (Tumlinson, 1977; Leal, 1996). This appears not to be the case in *N. vitripennis* since virgin females were also attracted by (4*S*,5*S*)-HDL, which is not released by males. (4*S*,5*R*)-HDL has been described among the fermentation products of *Streptomyces griseus* (Graefe et al., 1982). (+)Vernolic acid (9*Z*,12*S*,13*R*)-12,13-epoxyoctadec-9-enoic acid) has been shown to be the precursor of HDL stereoisomers in microorganisms (Garbe and Tressl, 2003). Further studies are in progress to investigate whether HDL is biosynthesized by the same pathway in insects and to characterize the pheromone-producing gland in the abdomen of *N. vitripennis* males.

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