CHAPTER 6

A Male Sex Pheromone in a Parasitic Wasp and Control of the Olfactory Response by the Female Mating Status

Abstract 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 nothing was known about the involved chemicals so far. Here we report the first identification of a male sex pheromone in parasitic Hymenoptera. In male abdomens of the jewel wasp *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 chemical signal. A neutral response was shown by mated females after 24 h and still after they had been allowed to oviposit for 6 d. *N. 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, since it increases the chance of insemination of virgins and decreases the probability for mated females to be disturbed by courting males when searching for new oviposition sites.

Key words *Nasonia vitripennis*, Pteromalidae, 5-hydroxy-4-decanolide, sex pheromone, mating.

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 primarily on their ability to locate and inseminate receptive females. Like in most other insects, sex pheromones are supposed to be of substantial importance during the mate finding process (Godfray, 1994). However, only a few chemicals have been identified in parasitic wasps so far (Kainoh, 1999; Ayasse et al., 2001; Keeling et al., 2004). These are exclusively female-derived compounds which are either highly volatile and attract males over long distances (Eller et al., 1984; Swedenborg and Jones, 1992; Swedenborg et al., 1994) or chemicals of low volatility and mediate arrestment and male courtship behaviour at close range (Shu and Jones, 1993; Syvertsen et al., 1995; Sullivan, 2002; Steiner et al., 2005, 2006). Nothing is known about the chemistry of male-derived sex pheromones in parasitic wasps, although a few behavioural studies suggest that they exist (van den Assem et al., 1980b; Gonzalez et al., 1985).

The physiological state of insects can have drastic impacts on their reproductive behaviour. When ejaculating males do not only transfer sperm but also a number of bioactive molecules from their accessory glands that induce a variety physiological and behavioural changes in females. Numerous studies (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 first evidence that the female mating status may also influence the responsiveness to olfactory stimuli. After mating or injection of accessory gland fluid, the female preference switched from the male sex pheromone to host odours (Jang, 1995, 2002).

In the present study, we report the identification of a male sex pheromone in the jewel wasp *Nasonia vitripennis* (Walker) (Pteromalidae), a model organism for the study of parasitic wasp biology, developing in puparia of numerous cyclorraphous fly species. 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 the male parasitoids. Furthermore, we determined release dynamics of individual males and studied whether the mating status of females influences their responsiveness to the male signal.

Methods and materials

Insects *Nasonia vitripennis* wasps were collected from a bird's nest in Northern Germany. Laboratory cultures were kept at 25°C and 60% relative humidity with a daily light:dark cycle of 16:8 h. Thirty to forty freshly emerged *N. vitripennis* 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 d. Parasitoid pupae were removed from host puparia 1-2 d 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 height, 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. A third group of mated females was allowed to parasitise fresh host pupae for 6 d before the bioassay.

Pheromone extraction and fractionation Unmated males of different age [< 1 h (N = 26), 1 d (N = 19), 2 d (N = 17), 3 d (N = 15)] and 1 d-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 individually for 30 min with five µl of dichloromethane containing 50 ng/µl methyl undecanoate (Sigma-Aldrich, Steinheim, Germany) as an internal standard. To narrow down the site of pheromone storage, 3-d-old unmated males (N = 5) were dissected with a scalpel into head, thorax and abdomen and tagmata were extracted separately as described above. Aliquots (1 µl) were analysed by GC-MS. Individual amounts of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL) 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. Batches of 20 3-d-old males were extracted for 30 min with 100 µl of dichloromethane. After removal of

the primary extract, the cadavers were washed twice with 100 µl dichloromethane. The combined extracts were applied to a silica gel cartridge (25 mg, International Sorbent Technology, Glamorgan, UK) and rinsed twice with 250 µl dichloromethane. The diastereomers of HDL were eluted with 250 µl methanol. After removal of the methanol under a gentle stream of nitrogen the residue was resolved 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 a commercially available adsorption filter (65 mm length x 5 mm diameter) designed for closed loop stripping analysis (CLSA) and equipped with a 1 mm charcoal layer (5 mg) for volatile adsorption (Gränicher & Quartero, Daumazan, France). Insects (3 d-old males, *N* = 12) were directly released into the filter tube and the open end of the tube was connected to a another glass tube (75 mm length x 6 mm diameter) containing 150 mg of charcoal to clean the air stream before entering the CLSA-tube. An air stream of 40 ml/min was sucked through the tube system for 5 h. Adsorbed volatiles were eluted with 25 µl of dichloromethane containing 5 ng/µl methyl undecanoate as an internal standard and used for chemical analysis by coupled gas chromatography-mass spectrometry (GC-MS). Amounts of HDL-diastereomers per sampling 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 x 1 h). Thereby, it was possible to estimate the HDL amounts released by individual males for each hour separately.

GC-MS analysis Extracts were subjected to GC-MS analysis on a Fisons 8060 GC (Fisons Instruments) equipped with a 30 m x 0.32 mm ID x 0.25 µm film thickness DB-5ms column (J & W Scientific, Folsom, CA, USA) operated in splitless mode (injector temperature: 240°C) and coupled to a Fisons MD 800 quadrupole MS running in the electron impact (EI) mode at 70 eV. Helium was used as carrier gas at a head pressure of 5 kPa (flow rate, 1.0 ml/min). Initial oven temperature was 80°C, increased at 5°C/min to 280°C and held for 30 min. Linear retention indices (LRI) of the male-specific double peak in the natural extracts were estimated by co-injection of a mixture of *n*-alkanes with chain lengths between 7 and 30 carbon units (Sigma-Aldrich, Steinheim, Germany). The compounds eluting at LRI = 1592 and

1608 were identified as a pair of diastereomers of HDL by comparison of mass spectra and LRI with those of authentic reference chemicals which were synthesised as described elsewhere (Garbe and Tressl, 2003). The two threo-enantiomers of HDL, (4*R*,5*R*)- and (4*S*,5*S*)-, eluted before the erythro-enantiomers (4*R*,5*S*)- and (4*S*,5*R*)-. Threo- and erythro-enantiomers of HDL were separated on a chiral 30 m x 0.25 mm x 0.25 μm film thickness β-DEX 225 GC-column (= 25% 2,3-di-*O*-acetyl-6-*O*-TBDMS-β-cyclodextrin in polydimethylsiloxane, Supelco, Bellefonte, PA, USA). Hydrogen was used as carrier gas. The flow was adjusted to 25 cm/s 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 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*)- eluting before (4*S*,5*S*) whereas the erythro-enantiomers were totally resolved with (4*R*,5*S*)- eluting before (4*S*,5*R*)-. The chirality of the natural products was finally established to be (4*R*,5*R*)- and (4*R*,5*S*)- 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 a cylinder made of acrylic (1.5 cm height, 7 cm diameter) divided into four chambers by crosswise arranged vertical plates. The top of the cylinder was covered by a plastic gauze (mesh 0.1 mm) functioning as a walking arena for the parasitoids. A lid consisting of a plastic ring (4 mm height, 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 & Nagel, Düren, Germany) were placed in each of the four chambers underneath the walking arena. Five μl of fractions containing natural HDL diastereomers (representing one male equivalent, see above) 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 were applied to the filter paper of one chamber (test). To test whether the female response is enantioselective, we tested also 80 ng of either the naturally occuring (4*R*,5*R*)- or the (4*S*,5*S*)-enantiomer that does not occur in the insects (see results). In all experiments, equal amounts of 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 d after copulation with oviposition opportunity and unmated males, *N* = 20 for each type) were released individually into the arena. The time wasps spent in the sectors above the four chambers was recorded for 300 seconds using the computer software The Observer 3.0 (Noldus, Wageningen, The Netherlands). To avoid biased results due to possible side preferences of the parasitoids, the olfactometer was rotated 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 analysed 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 x 1 h by a Mann-Whitney *U* test. Relative amounts (%) released per h in the 5 x 1 h experiment were analysed for equal distribution by a chi-square contingency table. Residence time of males spent above the test and control chamber in the bioassays were compared by Wilcoxon-matched pairs test. Statistical analyses were done using Statistica 4.5 scientific software (StatSoft, Hamburg, Germany).

Results

Chemical analysis of solvent extracts from *N. vitripennis* males and females by GC-MS revealed a male-specific double peak 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-phase. The natural product had the absolute configuration (4*R*,5*R*) and (4*R*,5*S*), respectively (Fig. 2). Amounts of HDL were close to zero in freshly emerged males, increased within the first two days after emergence and remained at a constant level on day three (Fig. 3). The ratio of (4*R*,5*S*)-/(4*R*,5*R*)-HDL changed successively with increasing age of the males in favour of (4*R*,5*S*) ($F = 9.980$, $df = 3$, $P < 0.001$, one-way ANOVA).

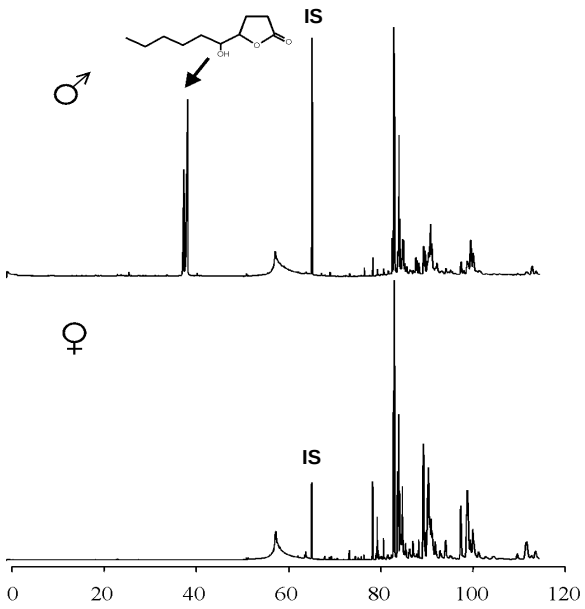

Fig. 1 Total ion current chromatograms of dichloromethane extracts from male and female *N. vitripennis* using a nonpolar DB-5ms capillary column. Arrow indicates the male-specific double peak belonging to diastereomers of HDL (IS = internal standard, 50 ng methyl undecanoate).

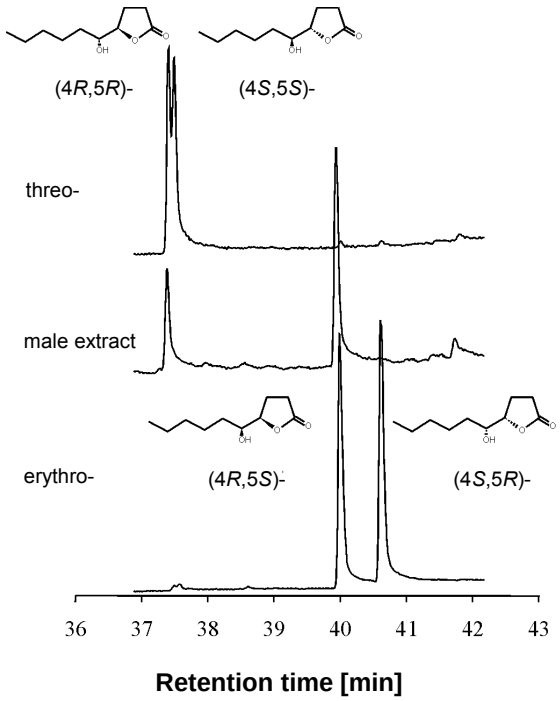

Fig. 2 Separation of threo- and erythro-enantiomers of HDL on a chiral β-DEX 225 GC-column. The natural product consists of (4*R*,5*R*)- and (4*R*,5*S*)-HDL.

Separate extraction of different body segments revealed that 99 ± 0.8 % (mean ± SE) of HDL was present in the abdomen and only trace amounts were detectable in extracts from head and thorax suggesting that the abdomen is the site of biosynthesis. After volatile sampling of individual males (5 h), HDL-diastereomers were recovered in total amounts of 80 ± 27 ng per male (mean ± SE). Total amounts released by individual males within 5 h were not significantly different from summed amounts released in the 5 x 1 h experiment (90.9 ± 20.4 ng per male, Mann-Whitney *U* test, $P = 0.391$). Eighty percent 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 ($\chi^2 = 1118.47$, $df = 32$, $P < 0.001$) (Fig. 4). Thereby, the time windows of pheromone release were highly variable for individual males.

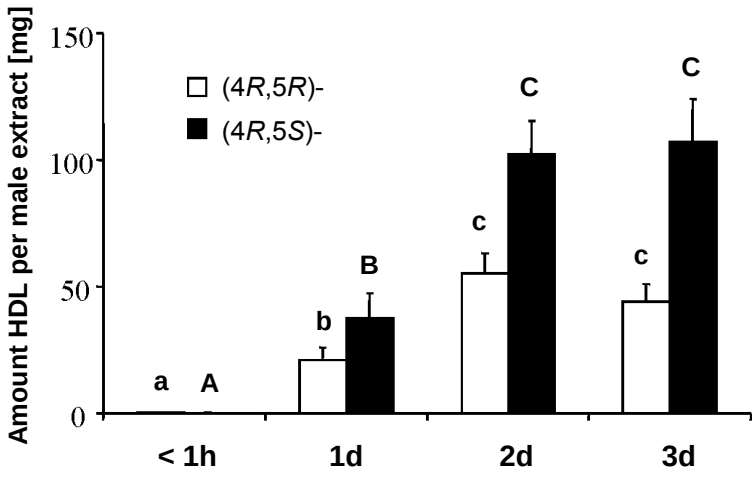

Fig. 3 Mean amounts (± SE) of HDL in whole body extracts from differently old males (different lower- and uppercase letters indicate significant differences for each diastereomer at $P < 0.05$, one-way ANOVA and LSD-test).

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) (Fig. 5), as well as by 80 and 160 ng of the synthetic mixture of (4*R*,5*R*)- and (4*R*,5*S*)-HDL. The response of virgin females was not enantioselective because both the naturally occurring (4*R*,5*R*)- as well as the (4*S*,5*S*)-enantiomer that does not occur in the parasitoids were attractive at a dose of 80 ng (Fig. 6). Five minutes after copulation mated females avoided one male

equivalent of natural HDL or 160 ng of the synthetic mixture (Fig. 5). A neutral response was shown by mated females after 24 h to all samples offered. Females that had been allowed to oviposit for 6 d responded neutrally to natural HDL, but there was a tendency to prefer the test field (P = 0.06).

Discussion

Mating systems of insects are often characterised by strong male-male competition for receptive females (Choe and Crespi, 1997). One strategy of male insects to increase their chance of successful reproduction is to release 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., 1980b; Gonzalez et al., 1985). In *N. vitripennis* both male and female pheromones have been reported. Female cuticular hydrocarbons release courtship behaviour in males (Steiner et al., 2006) who in turn elicit the readiness to copulate by releasing a still unknown aphrodisiac from their mandibular gland (van den Assem et al., 1980b). Furthermore, males are supposed to chemically mark territories after copulation. These marks are attractive for females and for other males (van den Assem et al., 1980b).

The present study reports the first identification of a male sex pheromone in parasitic Hymenoptera and adds another facet to pheromone communication in *N. vitripennis*: males synthesise HDL-diastereomers in their abdomen and release these chemicals to attract virgin females whereas males do not respond. *N. vitripennis* is a gregarious parasitoid developing in groups of 10 to 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 et al., 1980a). By releasing the sex pheromone, males may signal genetic quality and influence the outcome of this contest. In fact, males may downright “call” for females as indicated by the intermittent release of the pheromone even in the absence of females.

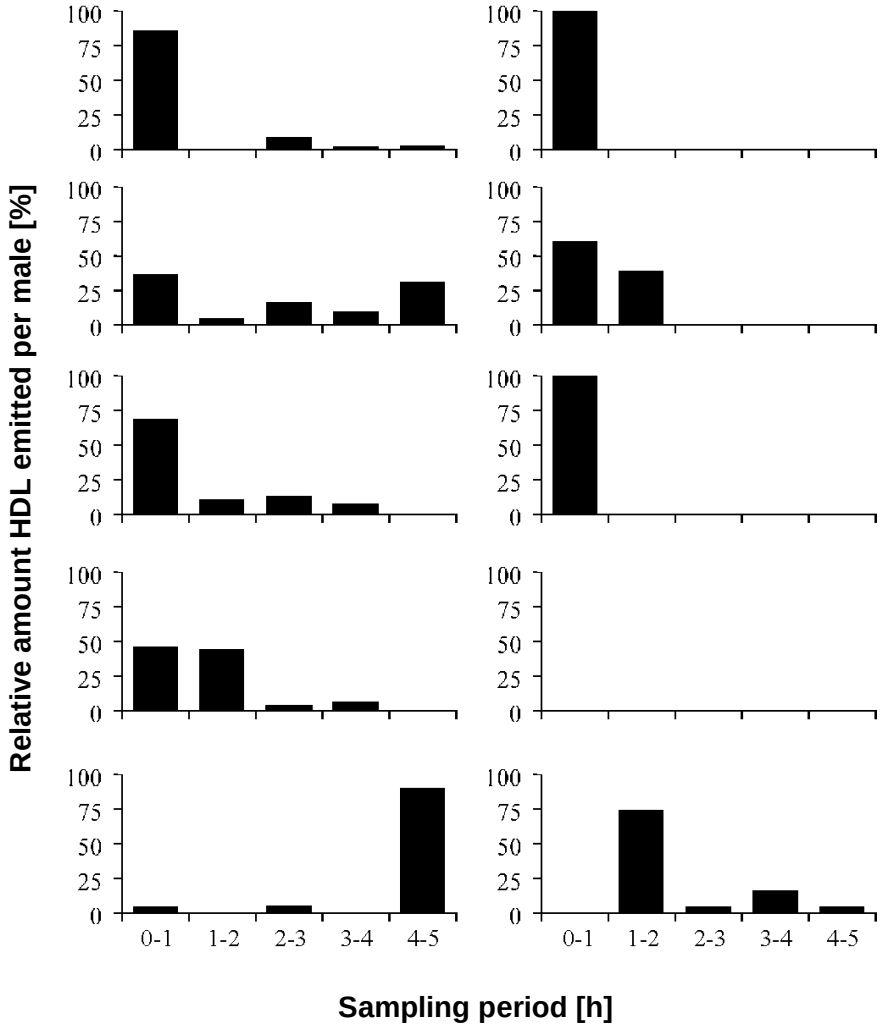

Fig. 4 Release dynamics of total HDL by individual *N. vitripennis* males during a 5 h sampling period (*N* = 10). Amounts released during each hour were related to the total amounts released within 5 h.

The present study clearly demonstrates that the responsiveness of females to the male sex attractant is under control of 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 olfactory preference switched from the male sex pheromone to host fruit odours (Jang, 1995, 2002). However, it was not distinguishable in the mentioned studies whether this switch was due to avoidance of the male pheromone or an increased attractiveness of the host fruit odour. 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.* However, refractoriness in *N. vitripennis* is supposed to be initiated during post-copulatory courtship behaviour. Van den Assem (1989) reported that an increase of remating events can be initiated in *N. vitripennis* females by preventing male post-copulatory behaviour. Thus, it remains to be tested whether also the olfactory switch in *N. vitripennis* females is associated to this behavioural element. Under normal circumstances, however, *N. vitripennis* females mate only once (van den Assem, 1989) and therefore, their variable response depending on the mating status makes sense from an evolutionary perspective: it increases the chance of immediate insemination of virgins and decreases the probability for mated females to be disturbed by courting males when searching for new oviposition sites.

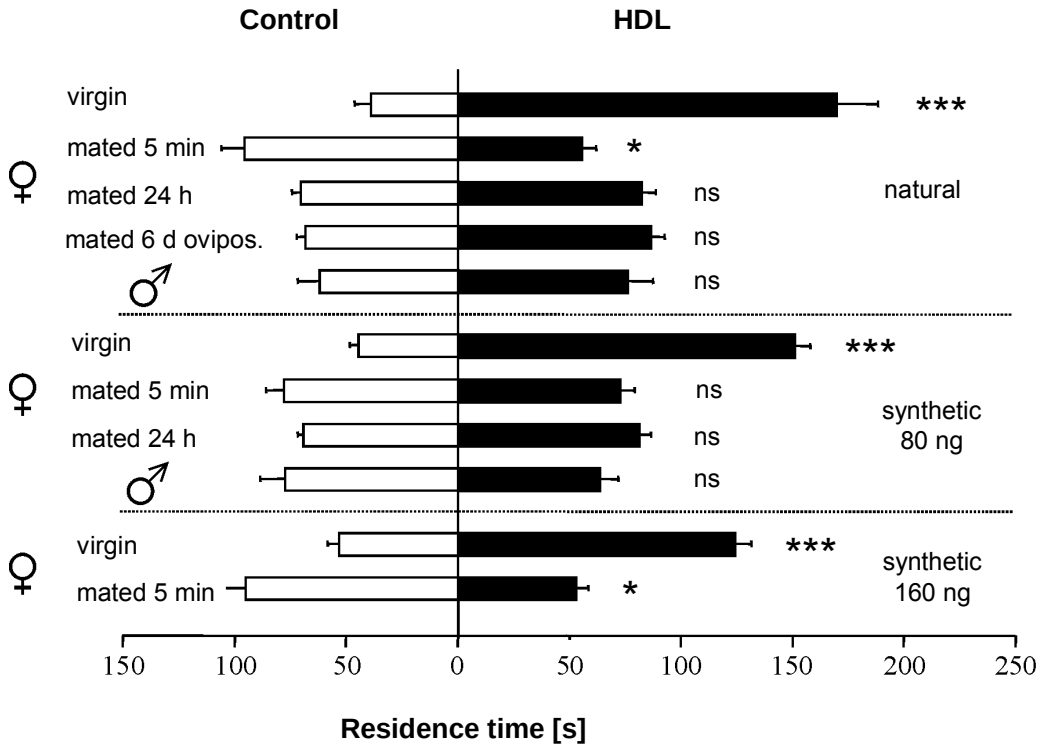

Fig. 5 Mean residence time (± SE) of females and males in odour and control fields of a two-choice olfactometer. Odour fields were treated with one individual male equivalent of natural HDL diastereomers or with synthetic (4*R*,5*R*)- and (4*R*,5*S*)-HDL (ratio 1:1.3) at doses of 80 or 160 ng. Control fields were treated with pure solvent (asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.001$ (***), ns = not significant (Wilcoxon matched pairs test, $N = 20$).

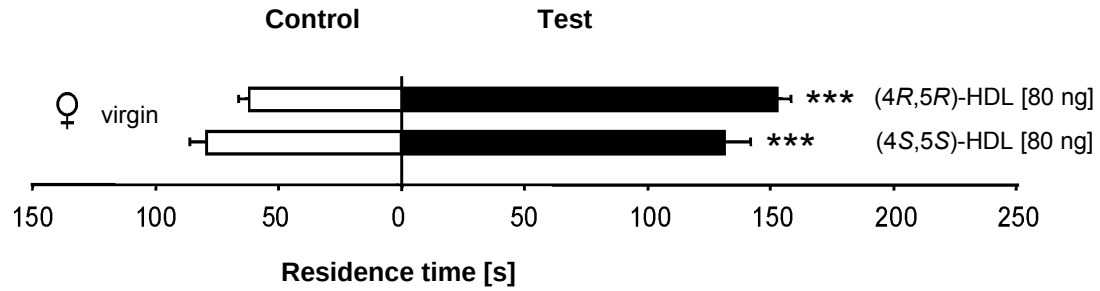

Fig. 6 Mean residence time of females in test and control fields of a two-choice olfactometer. Test fields were treated with (4*R*,5*R*)- or (4*S*,5*S*)-HDL at doses of 80 ng. Control fields were treated with pure solvent (asterisks indicate significant differences at $P < 0.001$ (***), ns = not significant (Wilcoxon matched pairs test, $N = 20$).

(4*R*,5*R*)- and (4*R*,5*S*)-HDL are reported here for the first time in insects although long-chain lactones including decanolides are common insect infochemicals (e.g., Howard et al., 1983; Nishida et al., 1996; Larsson et al., 2003). (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). Future studies will investigate whether HDL is biosynthesised by the same pathway in insects.

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