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The chemical basis of mate recognition in two parasitoid wasp species of the genus *Nasonia*

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Key words: parasitic wasp, chemical communication, cuticular hydrocarbons, pheromone, reproductive isolation, species discrimination, Hymenoptera, Pteromalidae

Abstract

To recognize one's mate is essential for all sexually reproducing animals. In insects, mate recognition is often based on chemical cues such as hydrocarbons which are distributed over the insect's cuticle. In the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae), interspecific mating possibly occurs in microsympatry between *Nasonia vitripennis* Walker and *Nasonia giraulti* Darling despite post-zygotic isolation mechanisms preventing hybridization. Males of *N. vitripennis* are known to equally court con- and heterospecific females, which they recognize by means of cuticular hydrocarbons. A recent study surprisingly showed that this might not be the case in *N. giraulti*, leaving open how males of this species achieve the recognition of mating partners. In this study, we investigated chemical mate recognition in *N. giraulti* in more detail and compared observed behaviors with behaviors of *N. vitripennis* by conducting experiments with both species concurrently and under the same experimental conditions. We disentangled the role of female-derived non-polar cuticular lipids – i.e., cuticular hydrocarbons – and more polar cuticular lipids in the ability of males to recognize con- and heterospecific females. In addition, we tested whether females of the two species discriminate similarly between con- and heterospecific males. We demonstrate that, in contrast to *N. vitripennis*, males of *N. giraulti* prefer live conspecific females over heterospecific ones. Furthermore, in contrast to *N. vitripennis*, mate recognition in *N. giraulti* males is not based on cuticular hydrocarbons, but rather involves other chemical messengers, presumably more polar cuticular lipids. In both species, discrimination against heterospecific males decreases with female age.

Introduction

Correct mate recognition is essential for all sexually reproducing organisms. For successful reproduction, a male encountering another individual needs to decide whether it belongs to the same species and the opposite sex. Reliable identification of potential mates can save time and energy otherwise invested in courtship and/or sperm transferred to the wrong recipients (Gröning & Hochkirch, 2008). Similarly, females need to decide whether the courting male belongs to the same species to avoid fitness costs due to fertilization by heterospecific sperm leading to unviable or no offspring, infertile hybrids, or hybrid breakdown in subsequent generations (Orr & Presgraves, 2000; Shapiro, 2000; Geuverink et al., 2009). As females usually invest more energy in the production of offspring than males, which is associated with higher costs of heterospecific mating, mate discrimination is expected to be stronger in females than in males (Trivers, 1972). This is particularly important in species constellations in which females mate only once and post-mating isolation is complete (Liou & Price, 1994). Although females should be choosier than males, mate discrimination by either sex can act as an effective pre-mating reproductive isolation mechanism to avoid costs of heterospecific mating.

In insects, recognition processes are widely based on chemical cues or signals (Wyatt, 2014). Chemical stimuli perceived by direct contact in insects are the cuticular hydrocarbons (CHCs; Singer, 1998). Besides functioning as an effective barrier preventing insects from desiccation

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(Gibbs, 1998), CHCs evolved into serving a plethora of functions concerning communication between and within insect species (Blomquist & Bagnères, 2010). Information encoded by CHCs include species, sex, and mating status in various insects as well as group membership, age, dominance, and fertility status in social Hymenoptera (Howard, 1993; Singer, 1998; Howard & Blomquist, 2005; Blomquist & Bagnères, 2010). In parasitic wasps, CHCs have been shown to serve as contact sex pheromones, which allow males to address courtship to adequate mating partners (Syvertsen et al., 1995; Ruther et al., 2000, 2011; Sullivan, 2002; Ablard et al., 2012; Ruther, 2013).

In the parasitoid wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae), female-derived CHCs elicit courtship behavior in males, whereas male-derived CHCs do not (Steiner et al., 2006). Males of *N. vitripennis*, however, do not discriminate between con- and heterospecific females and engage equally in mounting and courtship activities with females of different *Nasonia* species (Giesbers et al., 2013; Buellesbach et al., 2014). Females of *N. vitripennis* on the other hand mate more often with con- than with heterospecific males (Bordenstein & Werren, 1998; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014). A possible mechanism for females to recognize conspecific males is the males' courtship behavior, which consists of a stereotypic sequence of distinct behavioral elements. These include mounting the female and bouts of head-nodding behavior – i.e., moving of the male mouthparts along the female's antennae – coupled with the transfer of a male aphrodisiac pheromone, eventually leading to female receptivity and copulation (van den Assem et al., 1980; Ruther et al., 2010). After copulation, males perform post-copulatory courtship including additional series of head-nodding behavior. A detailed analysis of the courtship behavior in the four *Nasonia* species revealed some subtle species-specific differences (van den Assem & Vernel, 1979; van den Assem et al., 1980, 1981; van den Assem & Werren, 1994). Although not completely strict, the females' choosiness has the potential of playing a major part in maintaining prezygotic reproductive isolation between sympatric *Nasonia* species. Choosiness is especially important for females, as they usually mate only once before switching to host-seeking behavior (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014).

The genus *Nasonia* comprises four species all developing on pupae of cyclorrhaphous flies (Whiting, 1967; Darling & Werren, 1990; Raychoudhury et al., 2010a). All four species are gregarious parasitoids, i.e., females lay more than one egg into a fly puparium. After hatching, larvae feed as ectoparasites on the fly pupa inside the fly puparium, and emerge from the host after eclosion as adults. Except for *Nasonia giraulti* Darling and *Nasonia oneida* Raychoudhury & Desjardins, the *Nasonia* species are reproductively isolated by cytoplasmic incompatibility resulting from infections with different strains of the intracellular bacterium *Wolbachia* (Bordenstein et al., 2001). As a consequence, eggs fertilized by heterospecific sperm develop into male offspring and no viable hybrids are produced (Breeuwer & Werren, 1990).

Nasonia vitripennis is cosmopolitan and frequently occurs in microsympatry, i.e., within the same host individual, with each of the other three species, including *N. giraulti* in the eastern part of North America (Darling & Werren, 1990; Grillenberger et al., 2009; Raychoudhury et al., 2010a,b). In all *Nasonia* species, mating typically occurs at the natal host patch, from which mated females, but not males, disperse (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014). Microsympatry, thus, possibly leads to interspecific mating. As a result, a clear discrimination between con- and heterospecific mating partners would be expected in all *Nasonia* species to maintain species integrity and avoid costs imposed by interspecific mating.

In contrast to *N. vitripennis*, the chemical basis of mate recognition in the other three *Nasonia* species is not well understood yet. Two recent studies showed surprisingly that males of *N. giraulti*, confronted with dead females, preferred *N. vitripennis* females over conspecific ones (Buellesbach et al., 2013; Giesbers et al., 2013). The same preference was observed, when complete cuticular lipid extracts (hexane extracts) of female wasps were applied to washed wasp cadavers (so-called dummies; Buellesbach et al., 2013). These results pointed to a seemingly non-adaptive preference for heterospecific over conspecific mating partners in *N. giraulti* males, which is somewhat difficult to explain from an evolutionary perspective. The findings also raise questions relating to observations from other studies, where *N. giraulti* males readily courted and mated con- and heterospecific females equally (live females: Buellesbach et al., 2014; dead females: Ruther et al., 2014). A shift from chemical communication to other modes of communication, e.g., via behavioral, tactile, or acoustic cues, has been suggested for *N. giraulti* (Buellesbach et al., 2013). In contrast to *N. vitripennis* which mate on top of or in close vicinity to the host puparium after emergence, wasps of *N. giraulti* usually mate inside the host puparium prior to emergence, a behavior termed within-host mating (WHM; Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). Buellesbach et al. (2013) referred to the high WHM rates of up to 100% observed in *N. giraulti*, and hypothesized that these might render chemical messengers to recognize conspecific females unnecessary. However,

even if mating occurs inside the host puparium, *N. giraulti* males need to recognize females and, given the confined space and low light levels inside the puparium, the use of chemical messengers seems to be very likely. Therefore, the role of chemical stimuli in the process of mate recognition needs further clarification in this species.

Here, we investigate the chemical basis of mate recognition behavior in *N. giraulti* in more detail and compare the behavior of *N. giraulti* with that of *N. vitripennis* by conducting concurrent behavioral bioassays with both species. Previous studies on the chemical basis of mate recognition in *N. giraulti* did not differentiate clearly between complete cuticular lipid extracts and fractions of extracts gained from purification procedures (Buellesbach et al., 2013; Giesbers et al., 2013). Irrespective of the solvent used for extractions, complete cuticular lipid extracts may contain more polar lipids, such as free fatty acids, alcohols, glycerides, sterols, aldehydes, or ketones, in addition to the non-polar lipids, i.e., CHCs (Lockey, 1988; Buckner, 1993; Kühbandner & Ruther, 2015). Furthermore, polar cuticular lipids have been shown to function as pheromones in various insects (Buckner, 1993; Yew et al., 2009; Kühbandner et al., 2012). In this study, we therefore differentiate between complete cuticular lipid extracts and the non-polar lipid fraction containing the CHCs. Moreover, we avoid possible effects of inbreeding resulting from long-term laboratory rearing and effects of antibiotics treatment using two genetically diverse strains, namely NvHVRx and NgMix (van de Zande et al., 2014; Giesbers, 2016). In a stepwise reduction in information content available for recognition and discrimination, we performed a set of behavioral experiments in which males were confronted with live females, dead females, complete cuticular lipid extracts of females, or non-polar CHC fractions of these extracts alone. Differences in the cuticular lipid composition between females of the two strains were furthermore investigated by coupled gas chromatography-mass spectrometry (GC-MS). In addition, we tested female mate discrimination, taking into account possible age effects in female choosiness. Younger (0-day old) females of *N. vitripennis* are known to exhibit stronger discrimination against heterospecific males than older (2-day old) females (Ruther et al., 2014). This age effect has not been shown for *N. giraulti* females yet. By performing bioassays with both species concurrently and under the same experimental conditions, direct comparisons between the two species were possible.

We hypothesized that both *N. giraulti* and *N. vitripennis* males recognize conspecific females and that this recognition process is, at least partially, based on female-derived chemical stimuli. We furthermore predicted that, if males of *N. giraulti* discriminate between con- and heterospecific females, they prefer conspecific ones. For *N. vitripennis*, we predicted that males do not discriminate between con- and heterospecific females. Finally, we hypothesized that, similar to *N. vitripennis*, females of *N. giraulti* discriminate between con- and heterospecific males and that there is an age effect in this choosiness in both species.

Materials and methods

Rearing conditions and preparation of wasps

All experiments were performed with the two outbred laboratory strains of *N. vitripennis* and *N. giraulti*, NvHVRx and NgMix (van de Zande et al., 2014; Giesbers, 2016). Wasps were reared on *Lucilia caesar* (L.) (Diptera: Calliphoridae) pupae at 25 °C and L16:D8 photoperiod. In each generation, hosts were mixed 7 days after oviposition according to the rearing procedure described in van de Zande et al. (2014). To control for adult wasp age, wasp pupae were isolated from fly puparia 1–2 days before expected emergence. Single individuals were then kept in 1.5-ml microcentrifuge tubes and emergence was checked every morning. On emergence day, wasps were considered 0 days old. All experiments were done with 2-day-old females (or extracts thereof, except in experiment 1) and 0- to 4-day-old males. Assignment of wasps to treatments was randomized.

Experiment 1: Behavioral observations of living couples

We tested whether males of *N. vitripennis* and *N. giraulti* discriminate between live con- and heterospecific females. In addition, we tested whether females discriminate between males of the two species when courted and whether there is an age effect in female choosiness. Males of *N. vitripennis* and *N. giraulti* were tested in single confrontations with 0- and 2-day-old females of both species presenting females singly and resulting in eight different treatments (n = 20 per treatment). For observations we used, a mating arena consisting of a round hole (1 cm diameter) cut in a 3-mm-thick sheet of acrylic glass. The arena was put on a piece of filter paper and closed by a glass slip after introduction of a female and a male. Behaviors were recorded with a Sony Nex-5 camera connected to a stereo microscope (Schott, KL 2000 LED) at 10× magnification. Recording lasted until the end of post-copulatory courtship. Recording was stopped if mating did not occur within 5 min. Movies were analyzed at half-speed using the video module of The Observer XT v.11.5 software (Noldus Information Technology, Wageningen, The Netherlands). The time from first antennal contact to when the male mounted the female (first contact to mounting), the time from mounting to the start of head-

nodding behavior (mounting to head-nodding), the duration of the copulation (duration of copulation), and the duration of post-copulatory courtship (post-copulatory courtship) were recorded. These behaviors were considered to be possible indicators of male mate discrimination which are not influenced by the behavior of the female. In addition, we recorded the duration of head-nodding behavior (duration head-nodding) and whether copulations occurred or not (copulations). These two parameters were considered to be indicators for female mate choice. A short duration of head-nodding was considered to represent increased female interest in the specific male because head-nodding is terminated by the female's receptivity signal (head lowering and opening of the genital pouch).

Experiment 2: Response of males to dead females

Dead females were presented to males to test whether males are able to recognize and/or discriminate between females excluding effects of female behavior, such as receptivity signaling or movements. Dead females were presented either separately in single confrontations, or two females were presented in simultaneous confrontations (one of each species). In addition, a negative control was set up with solvent-washed females presented to males in single confrontations to check for the involvement of chemical stimuli in mate recognition (eight treatments: males of both species tested in single confrontations with females of either species and control, plus males of both species tested in simultaneous confrontations; n = 20 per treatment). Two-day-old *N. vitripennis* and *N. giraulti* females were killed by freezing and kept at −20 °C until being used but no longer than 3 days. For the control, dead females from both species were pooled and washed continuously with dichloromethane (DCM) (Roth, Karlsruhe, Germany) for 6 h using a Soxhlet extractor. To ensure that all extractable chemicals had been removed from these females, they were extracted again for 10 min in 20 µl DCM per individual and extracts were analyzed by GC-MS. After evaporation of the solvent residues, dead females were glued on pieces of filter paper (55 mm diameter) and presented to males in the middle of the mating arena. In simultaneous confrontations, two females, one from each species, were glued next to each other with their heads pointing into the same direction. Positions of the two species were exchanged after every replicate. Video recording and behavioral analysis was similar to experiment 1 and lasted for 5 min after the test male's first antennal contact with a dead female. Behaviors recorded were: the time the male spent mounted on the female (time mounted) and the number of copulation attempts (copulation attempts). This procedure was the same in all further experiments.

Experiment 3: Response of males to female-derived complete cuticular lipid extracts

Complete cuticular lipid extracts of females (solved in DCM) were applied on dummies and presented to males in single confrontations to test whether males are able to recognize and/or discriminate between females based on chemical messengers only, excluding female-specific visual and tactile cues. As a control, pure DCM was applied (six treatments: males of both species tested with extracts of females of either species and control; n = 20 per treatment). To avoid possible effects of female-specific cuticular structures and visual cues on male responses, solvent-washed males were used as dummies in this and the following experiment. For dummy preparation, males of both species were killed by freezing, pooled and washed 3× for 10 min in 20 µl DCM per individual. The absence of cuticular lipids was confirmed by GC-MS analysis. For extract preparation, 40–100 dead 2-day-old females were pooled and extracted for 10 min in 20 µl DCM per individual. Complete cuticular lipid extracts were then concentrated to a final concentration of two female equivalents per µl DCM. Single randomly chosen male dummies were glued to filter papers and 1 µl of the extract to be tested was applied to each dummy by means of a 5 µl microsyringe (Hamilton, Bonaduz, Switzerland). Extracts were carefully applied to the dummies in small steps to avoid leakage of the extract to the filter paper. Dummies were tested in the bioassays between 30 min and 4 h after extract application. Bioassays and data collection followed the procedures described for experiment 2.

Experiment 4: Response of males to female-derived cuticular hydrocarbon fractions

Complete cuticular lipid extracts were fractionated and the non-polar CHC fractions were tested alone in single confrontations to test whether CHCs alone are sufficient for the recognition of and/or discrimination between females. By this procedure, more polar cuticular lipids such as cholesterol, triacylglycerides (TAGs), and free fatty acids were excluded. Pure DCM was used as a control (six treatments, n = 20 each). To isolate the CHCs, complete cuticular lipid extracts of both species (representing 50–100 female equivalents) were dried under a stream of nitrogen and re-dissolved in 200 µl hexane (Roth). Extracts were then applied to 100 mg SiOH cartridges conditioned by rinsing with hexane, and CHCs were eluted by washing cartridges twice with 200 µl hexane. The absence of oxygenated lipids was confirmed by GC-MS analysis. Prior to use, the solvent was evaporated again and the CHCs were re-dissolved in DCM to a concentration of two female equivalents per µl. Dummy preparation, bioassays and

data collection followed the procedures described for experiment 3.

Chemical composition of female-derived cuticular lipids

The composition of compounds in extracts from *N. vitripennis* and *N. giraulti* females was analyzed by GC-MS. Two-day-old females of both species were killed by freezing. For each sample, two females were pooled and extracted for 10 min in 25 µl of DCM containing 10 ng μl^{-1} tetracosane (C24) as an internal standard (n = 10 per species). Extracts were analyzed on a Shimadzu QP2010 Plus GC-MS system equipped with a BPX-5 capillary column (30 m × 0.25 mm, 0.25 µm film thickness; SGE, Milton Keynes, UK). Aliquots of 2 µl of each extract were injected splitless at 300 °C by means of a Shimadzu AOC 20i autosampler. Oven temperature started at 150 °C, increased by 3 °C per min and was held at 300 °C for 20 min. Helium was used as carrier gas at a linear velocity of 50 cm s^{-1}. The interface temperature was 300 °C. Substances were ionized by electron impact ionization (EI) at 70 eV. Ion source temperature was 200 °C. An n-alkane mixture (C_7-C_{40}) was analyzed under the same conditions to determine the relative retention indices (RIs) (van den Dool & Kratz, 1963). Compounds were identified by means of comparison of their RIs with literature data (Steiner et al., 2006; Niehuis et al., 2010; Buellesbach et al., 2013) and by the analysis of diagnostic ions in the mass spectra.

Statistical analysis

Differences in the responses of males toward conspecific and heterospecific females from experiment 1 (live couples) were analyzed by two-tailed Mann–Whitney U-tests. In the analysis of 'duration of copulation' and 'post-copulatory courtship', only samples with successful copulations were included. Data from experiment 1 on behaviors connected to female mate discrimination were first tested for differences in the response toward con- and heterospecific males, pooling data of bioassays with 0- and 2-day-old females. Subsequently, we tested for age effects within con- and heterospecific couples separately. Data on mating rates (number of females consenting to mating) were analyzed by 2 × 2 χ^2 tests. Differences in the duration of head-nodding behavior were analyzed by Mann–Whitney U-tests including only data from samples with successful copulations. As we expected females to discriminate against heterospecific males, and as we expected discrimination to be stronger in older females than in younger ones, one-tailed tests were performed. The time males spent mounted on dead females in simultaneous confrontations (experiment 2) was analyzed by Wilcoxon signed rank tests for paired samples. Data from single confrontations with dead females (experiment 2) and from experiments with dummies (experiments 3 and 4) were analyzed by Kruskal–Wallis H-tests followed by pairwise comparisons using Bonferroni-corrected Mann–Whitney U-tests. Two-tailed tests were performed for comparisons between con- and heterospecific stimuli, and one-tailed tests were performed for comparisons with washed females and controls. All non-parametric tests were performed for *N. vitripennis* and *N. giraulti* separately.

To compare the responses between the two species and between stimuli with differing information contents (comparing experiments 2, 3, and 4), additionally generalized linear models (glms) were performed, because these allow the implementation of more complex designs including interactions. For experiment 1, a separate model was calculated for each recorded behavior. Mating frequencies were analyzed by fitting a logistic regression model (binomial distribution with logit link function). All other behaviors from experiment 1 were analyzed by fitting models based on the proportion of time spent exhibiting the respective behavior, with logit link function and assuming pseudo-binomial error structure (Faraway, 2016). Species, type of pairing (con- or heterospecific), and—in the case of mating frequencies and 'duration head-nodding'—female age were included as fixed factors. The proportion of time spent mounted on dead females or dummies in experiments 2 (only single confrontations), 3, and 4 were analyzed together by fitting a glm with logit link function and pseudo-binomial error structure, and the corresponding numbers of males showing copulation attempts were analyzed by fitting a logistic regression model. Species, type of pairing, and information content (dead females, complete cuticular lipid extract, CHC fraction) were included as fixed factors. For each model, factors and interactions not contributing significantly to the fit were dropped by a stepwise reduction in the model using the drop1 function in R.

The composition of female-derived cuticular lipids was investigated by multivariate analysis of the relative amounts of single compounds (or compound mixes in unresolved peaks). Relative amounts of single peaks were calculated by relating single peak areas to the area of the peak representing the internal standard. Peaks which represented on average less than 0.05% of the total amount of compounds per sample were neglected. A data matrix containing the relative amounts of all peaks per sample was set up. Differences in the lipid composition between the two species were analyzed by a type II PERMANOVA (permutational multivariate analysis of variance; Anderson, 2001) with 9 999 permutations using Bray-Curtis dissimilarities as distance measure. In addition, a SIMPER (similarity percentage)

analysis was performed to detect peaks essential for the observed differences. Total amounts of cuticular lipids (in ng per individual) were calculated based on the internal standard peak area representing 20 ng of substance, and compared between *N. vitripennis* and *N. giraulti* females by means of a Mann–Whitney U-test. Moreover, differences in relative amounts of summed n-alkanes, monomethyl-, dimethyl-, trimethyl- and tetramethylalkanes were assessed. For graphical display, a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarities was drawn.

All analyses were done in R v.3.2.0 (R Development Core Team, 2015). An α-level of 0.05 was chosen as significance level for statistical tests.

Results

Experiment 1: Behavioral observations of living couples

Both *N. vitripennis* and *N. giraulti* males courted con- and heterospecific live females. In all behaviors considered to be possible indicators of male choosiness, *N. vitripennis* males did not differentiate between females of the two species (Mann–Whitney U-test, 1st contact to mounting: $P = 0.23$; mounting to head-nodding: $P = 0.30$; duration of copulation: $P = 0.82$; post-copulatory courtship: $P = 0.10$; Figure 1A, C, E, and G). *Nasonia giraulti* males took more time to mount females and to start head-nodding when exposed to heterospecific as compared to conspecific ones (Mann–Whitney U-test, 1st contact to mounting: $P = 0.01$; mounting to head-nodding: $P = 0.04$; Figure 1B and D). No significant differences were observed in the durations of copulation and post-copulatory courtship in *N. giraulti* (Mann–Whitney U-test, duration of copulation: $P = 0.07$; post-copulatory courtship: $P = 0.09$; Figure 1F and H). In general, *N. giraulti* males spent more time on post-copulatory courtship than *N. vitripennis* males (glm: $F_{1,111} = 87.3$, $P<0.0001$; Table S1).

In both species, females discriminated between con- and heterospecific mating partners. In general, females

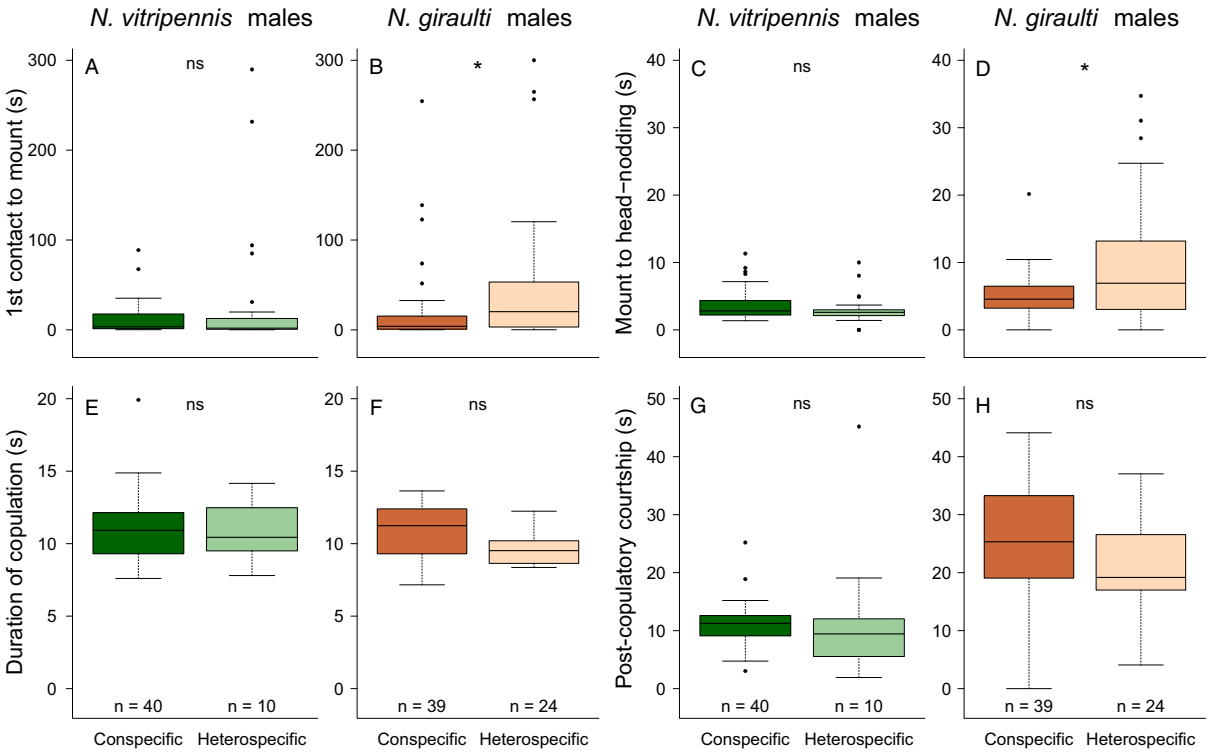


Figure 1 Box plots of behaviors associated with (A, C, E, G) *Nasonia vitripennis* and (B, D, F, H) *N. giraulti* male mate discrimination in bioassays with live con- and heterospecific couples. The behaviors are: (A, B) time between first antennal contact and mounting the female, (C, D) time between mounting the female and starting courtship (head-nodding behavior; A–D: n = 20 each), (E, F) duration of copulation, and (G, H) duration of post-copulatory courtship (E-H: sample sizes are below the boxes). Boxes indicate median (horizontal line within the box), 25–75% quartiles (upper and lower box margins), and maximum/minimum range (whiskers), and the dots indicate outliers (>1.5× above/below box height). Asterisks indicate significant differences between con- and heterospecific couples (Mann–Whitney U-tests: $P<0.05$; ns, non-significant).

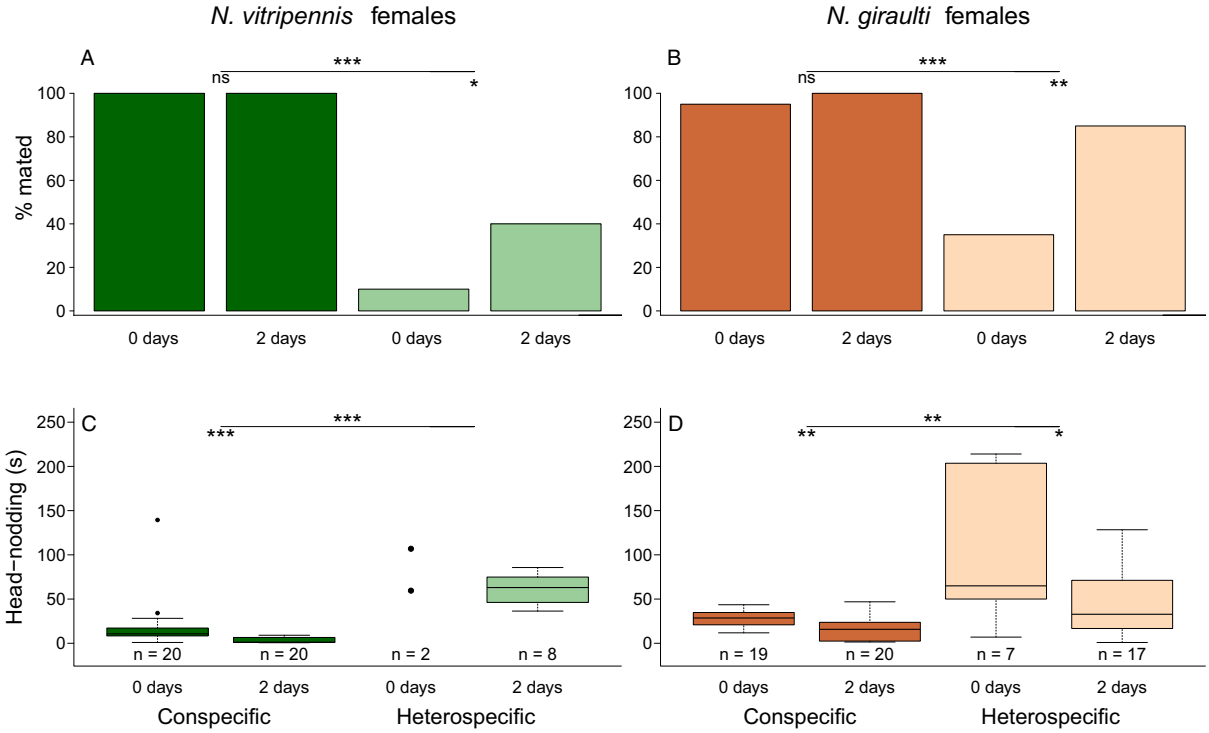


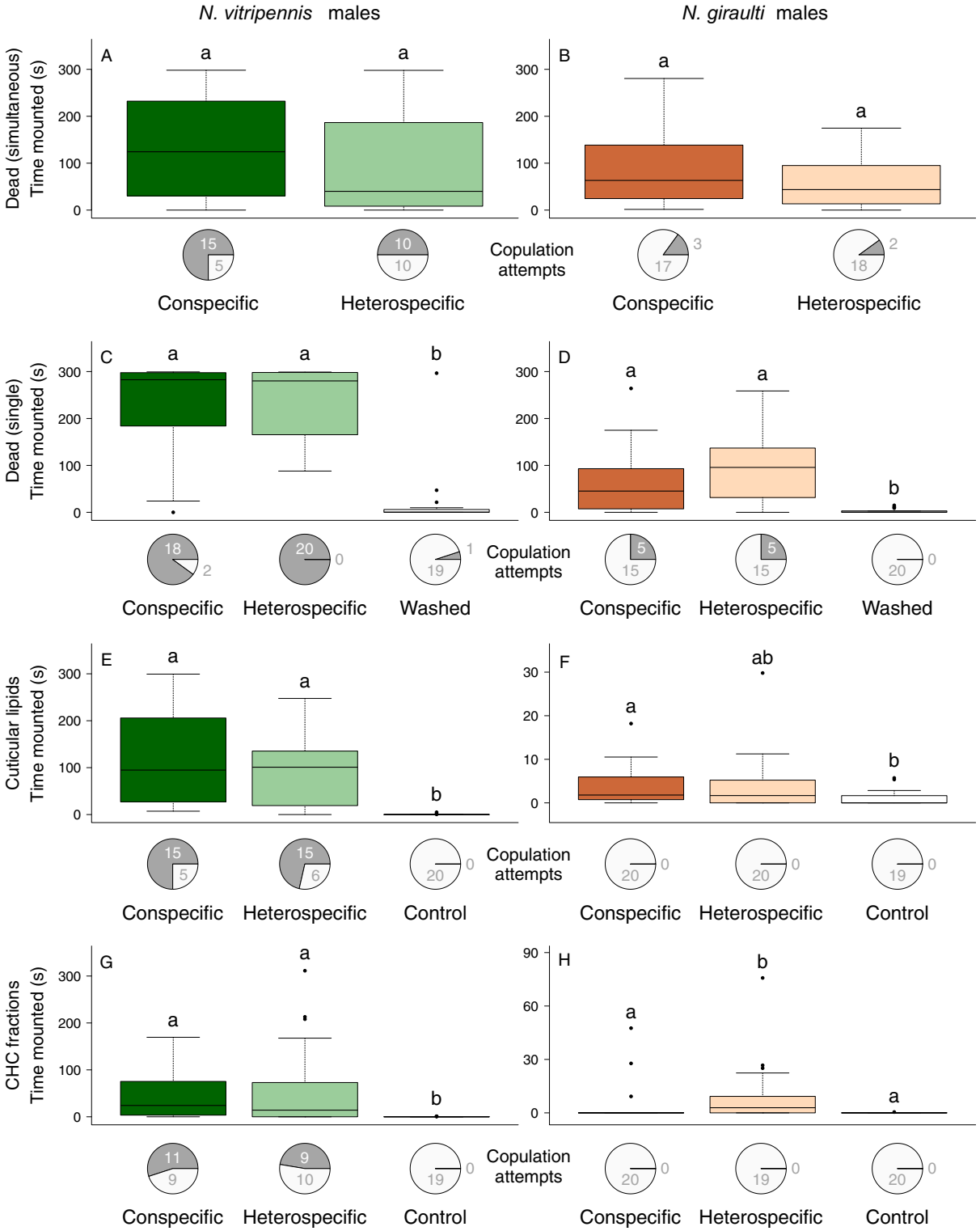
Figure 2 Behaviors associated with (A, C) *Nasonia vitripennis* and (B, D) *N. girault* female mate discrimination (displayed according to female age) in bioassays with live con- and heterospecific couples. The behaviors are: (A, B) percentage of females consenting to copulation (n = 20 each) and (C, D) duration of male courtship behavior (head-nodding) before consenting to copulation (sample sizes are below the boxes). Boxes in panels C and D indicate median (horizontal line within the box), 25–75% quartiles (upper and lower box margins), maximum/minimum range (whiskers), and the dots indicate outliers (>1.5× above box height). Asterisks indicate significant differences between con- and heterospecific couples (A, B, copulations: 2 × 2 χ^2 tests; C, D, head-nodding: Mann–Whitney U-tests: *0.01<P<0.05, **0.001<P<0.01, ***P<0.001; ns, non-significant).

mated more often with conspecifics than with heterospecifics (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 48$; *N. girault*, $\chi^2 = 16.8$, both P<0.0001; Figure 2A and B). Discrimination against heterospecific mates was stronger in *N. vitripennis* than in *N. girault* females (logistic regression model, % mated, species*type of pairing: $\chi^2 = 3.9$, P = 0.049; glm, head-nodding, species*type of pairing: $F_{1,108} = 4.6$, P = 0.03; Table S2). Prior to copulation, heterospecific males had to spend more time in head-nodding than conspecific males (Mann–Whitney U-test: *N. vitripennis*, P<0.0001; *N. girault*, P<0.01; Figure 2C and D).

In conspecific pairings, all females, except one 0-day-old *N. girault* female, became receptive. Two-day-old females became receptive faster than 0-day-old ones (Mann–Whitney U-test, head-nodding: *N. vitripennis*, P<0.001; *N. girault*, P<0.01). Also in heterospecific pairings, an age effect in female mate discrimination was found in both species, with 2-day-old females becoming receptive more often than 0-day-old ones (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 4.8$, P<0.05; *N. girault*, $\chi^2 = 10.42$, P<0.01). Due to the low number of females that consented to heterospecific mating in *N. vitripennis* (n = 2 and 8, for 0- and 2-day-old females, respectively), no statistical test was performed on the duration of head-nodding in this species. In *N. girault*, an age effect was found in the duration of head-nodding in heterospecific couples as well (Mann–Whitney U-test: P = 0.03).

Experiment 2: Response of males to dead females

In simultaneous confrontations, males of both species spent similar amounts of time on con- and heterospecific dead females (Wilcoxon signed rank test: P>0.3 for both species; Figure 3A and B). Copulation attempts occurred in both species as often with con- as with heterospecific dead females (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 2.67$, P = 0.10; *N. girault*, $\chi^2 = 0.23$, P = 0.63). Also in single confrontations, males of both species did not discriminate



between females of the two species but spent less time on solvent-washed dead females (Kruskal-Wallis test: $P<0.0001$; Bonferroni-corrected Mann–Whitney U-test pairwise comparisons: conspecific vs. heterospecific, $P>0.3$; conspecific vs. washed and heterospecific vs. washed, both $P<0.0001$; Figure 3C and D). Copulation

Figure 3 Box plots of the time (A, C, E, G) *Nasonia vitripennis* and (B, D, F, H) *N. girault*i males spent mounted on dead females (A, B) presented together (simultaneous confrontations) or (C, D) presented separately (single confrontations), and on dummies applied with (E, F) complete cuticular lipid extracts of females, or (G, H) with cuticular hydrocarbon (CHC) fractions of female extracts (n = 20 each). Boxes indicate median (horizontal line within the box), 25–75% quartiles (upper and lower box margins), and maximum/minimum range (whiskers), and the dots indicate outliers (>1.5× above/below box height). Washed: dead solvent-washed females; control: pure dichloromethane. Boxes within a panel capped with different letters indicate significant differences between treatment effects (Mann–Whitney U-tests: P<0.05). The pie charts indicate frequencies of samples with (gray) and without (white) copulation attempts.

attempts in single confrontations were observed as often with con- as with heterospecific dead females (2×2 χ^2 test: *N. vitripennis*, $\chi^2 = 2.11$, $P = 0.15$; *N. girault*i, $\chi^2 = 0$, $P = 1$). Only one *N. vitripennis* male showed copulation attempts toward a washed dead female.

Experiment 3: Response of males to female-derived complete cuticular lipid extracts

When exposed to complete cuticular lipid extracts, *N. vitripennis* males spent similar amounts of time on dummies treated with con- and heterospecific extracts, respectively, whereas the pure solvent was less attractive (Kruskal-Wallis test: P<0.0001; Bonferroni-corrected Mann–Whitney U-test pairwise comparisons: conspecific vs. heterospecific, P = 1; conspecific vs. control and heterospecific vs. control, both P<0.0001; Figure 3E). Copulation attempts were performed similarly often toward dummies treated with con- and heterospecific extracts (2×2 χ^2 test: $\chi^2 = 0.07$, $P = 0.8$), and no copulation attempts were observed toward the solvent control dummies. Likewise, *N. girault*i males did not discriminate between female-derived extracts of the two species, spent more time on dummies applied with extract of conspecifics than on pure solvent, but did not discriminate between solvent control and extracts of heterospecific females (Kruskal-Wallis test: P = 0.04; Bonferroni-corrected Mann–Whitney U-test pairwise comparisons: conspecific vs. heterospecific, P = 1; conspecific vs. control, P = 0.04; heterospecific vs. control, P = 0.18; Figure 3F). No copulation attempts were performed by *N. girault*i males in response to cuticular lipid extracts.

Experiment 4: Response of males to female-derived cuticular hydrocarbon fractions

When exposed to dummies treated with the non-polar CHC fraction, *N. vitripennis* males spent more time on female CHCs than on the solvent control and did not differentiate between CHCs of the two species (Kruskal-Wallis test: P<0.0001; Bonferroni-corrected Mann–Whitney U-test pairwise comparisons: conspecific

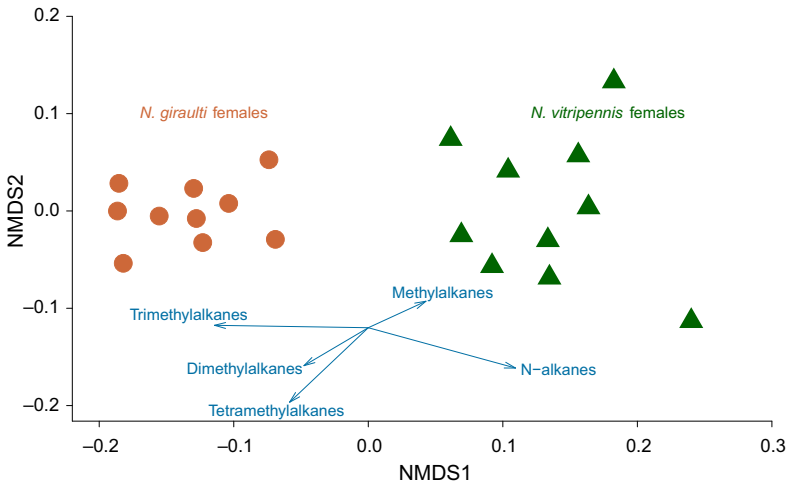


Figure 4 Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarities of relative amounts of chemical compounds found in dichloromethane (complete cuticular lipid) extracts of *Nasonia vitripennis* (triangles) and *N. girault*i (circles) females. Each data point represents one sample. The distance between data points represents the degree of chemical dissimilarity between samples. The contribution of various classes of hydrocarbons is represented by arrows, whose direction is based on the amount of the respective class, and whose length represents the intensity of the correlation.

Table 1 Relative amounts (mean ± SD) of compounds found in complete cuticular lipid extracts (dichloromethane extracts) of females of *Nasonia girault*i (strain NgMix) and *N. vitripennis* (strain NvHVRx)

Peak	RI	Compound names	Diagnostic ions	*N. girault*i	*N. vitripennis*	Type	Contr. (%)
1	2900	C29	408	1.26 ± 0.74	2.29 ± 0.64	n	3.47
2	2939	7-MeC29	407(M-51), 112/113, 336/337	0.44 ± 0.36	0.97 ± 0.32	Mono	1.81
3	3000	C30	422	0.62 ± 0.05	0.93 ± 0.12	n	0.9
4	3100	C31	436	16.35 ± 1.81	22.17 ± 2.45	n	16.5
5	3128	Cholesterol	368, 386	6.46 ± 0.67	6.46 ± 0.94	Mono	2.55
		15-MeC31	435(M-15), 224/225, 252/253				
		13-MeC31	435(M-15), 196/197, 280/281				
		11-MeC31	435(M-15), 168/169, 308/309				
		9-MeC31	435(M-15), 140/141, 336/337				
6	3140	7-MeC31	435(M-15), 112/113, 364/365	5.19 ± 0.48	8.33 ± 2.05	Mono	8.88
7	3150	5-MeC31	435(M-15), 84/85, 392/393	5.34 ± 0.52	3.71 ± 0.71	Di	4.58
		13,x-DiMeC31	449(M-15), 196/197, 294/295				
		11,x-DiMeC31	449(M-15), 168/169, 322/323				
		9,x-DiMeC31	449(M-15), 140/141, 350/351				
8	3165	x,x-DiMeC31	449(M-15)	0.95 ± 0.13	0.86 ± 0.11	Di	0.44
9	3174	3-MeC31	421(M-15)	9.49 ± 0.91	9.08 ± 1.76	Mono	4.24
10	3202	3,x-DiMeC31	449(M-15), 435(M-29)	1.09 ± 0.08	0.87 ± 0.09	Di	0.68
		Unknown HC					
11	3228	3,x,x-TriMeC31	449(M-29, 435(M-43)	1.15 ± 0.15	0.96 ± 0.11	Tri	0.64
		5,x,x-TriMeC31	449(M-29), 435(M-43), 84/85				
12	3248	3,7,11,15-TetraMeC31	477(M-15), 463(M-29), 126/127, 392/393, 196/197, 322/323, 266/267, 252/253	0.96 ± 0.19	1.03 ± 0.2	Tetra	0.6
13	3300	C33	464	1.1 ± 0.21	1.43 ± 0.27	n	1.11
		Unknown HC					
14	3326	17-MeC33	463(M-29), 252/253	11.66 ± 0.93	10.63 ± 1.46	Mono	4.44
		15-MeC33	463(M-15), 225/225, 280/281				
		13-MeC33	463(M-15), 196/197, 308/309				
		11-MeC33	463(M-15), 168/169, 337/338				
15	3338	7-MeC33	463(M-15), 112/113, 392/393	1.11 ± 0.2	1.53 ± 0.17	Mono	1.23
16	3352	5-MeC33	463(M-15), 84/85, 420/421	4.11 ± 0.67	4.11 ± 0.67	Di	5.33
		15,x-DiMeC33	477(M-15), 224/225, 294/295				
		11,x-DiMeC33	477(M-15), 168/169, 350/351				
17	3362	7,23-DiMeC33	477(M-15), 112/113, 406/407, 168/169, 351/352	0.79 ± 0.1	0.25 ± 0.14	Di	1.49
		7,19-DiMeC33	477(M-15), 112/113, 406/407, 224/225, 294/295				
18	3372	x,15,x-TriMeC33	491(M-15), 168/169, 364/365	6.79 ± 0.65	2.55 ± 0.54	Tri	11.92
		Unknown HC					
19	3398	3,x-DiMeC33	477(M-15), 463(M-29)	1.49 ± 0.2	1.22 ± 0.23	Di	1
		Unknown HC					
20	3427	5,9,21-TriMeC33	491(M-15), 477(M-29), 84/85, 449/450, 154/155, 378/379, 350/351, 196/197	1.26 ± 0.14	0.86 ± 0.17	Tri	1.16
		x,7,x-TriMeC33	491(M-15), 477(M-29), 126/127, 406/407				
21	3446	3,7,11,15-TetraMeC33	505(M-15), 491(M-29), 126/127, 420/421, 196/197, 350/351, 266/267, 280/281	4.51 ± 0.48	1.31 ± 0.28	Tetra	9.04
22	3465	Unknown HC		0.32 ± 0.1	0.07 ± 0.07	–	0.74
23	3524	17-MeC35	491(M-15), 252/253, 280/281	4.62 ± 0.45	6.64 ± 0.76	Mono	5.66
		15-MeC35	491(M-15), 224/225, 308/309				
		13-MeC35	491(M-15), 196/197, 336/337				

Table 1. Continued

Peak	RI	Compound names	Diagnostic ions	*N. girault*i	*N. vitripennis*	Type	Contr. (%)
24	3544	15,x-DiMeC35	505(M-15), 224/225, 322/323	5.54 ± 0.51	4.65 ± 1.12	Di	3.24
		13,x-DiMeC35	505(M-15), 196/197, 350/351				
		11,x-DiMeC35	505(M-15), 168/169, 378/379				
25	3560	7,23-DiMeC35	505(M-15), 112/113, 435/436, 280/281, 238/239	0.54 ± 0.12	1.42 ± 0.29	Di	2.54
		7,19-DiMeC35	505(M-15), 112/113, 435/436, 252/253, 294/295				
		7,15-DiMeC35	505(M-15), 112/113, 435/436, 196/197, 350/351				
26	3568	5,x-DiMeC35	505(M-15), 84/85, 462/463	3.25 ± 0.4	3.09 ± 0.41	Di	1.3
27	3596	3,15-DiMeC35	505(M-15), 491(M-29), 238/239, 308/309	0.28 ± 0.05	0.25 ± 0.12	Di	0.25
		Unknown HC					
28	3643	Unknown HC		0.45 ± 0.11	0.28 ± 0.12	–	0.54
29	3722	17-MeC37	519(M-15), 252/253, 322/323	0.64 ± 0.18	0.7 ± 0.26	Mono	0.7
		15-MeC37	519(M-15), 224/225, 350/351				
		13-MeC37	519(M-15), 196/197, 378/379				
30	3741	15,x-DiMeC37	533(M-15), 224/225, 322/323	1.07 ± 0.24	1.31 ± 0.2	Di	0.91
		13,x-DiMeC37	533(M-15), 196/197, 350/351				
		11,x-DiMeC37	533(M-15), 168/169, 406/407				
31	3758	7,x-DiMeC37	533(M-15), 112/113, 462/463	0.05 ± 0.08	0.05 ± 0.08	Di	0.83
32	3766	5,x-DiMeC37	533(M-15), 84/85, 490/491	1.09 ± 0.25	0.63 ± 0.18	Di	1.28

RI: retention index; type: assignment of peaks to hydrocarbon types for statistical analysis: n: n-alkane, Mono: monomethylalkane, Di: dimethylalkane, Tri: trimethylalkane, Tetra: tetramethylalkane. Contr.: contribution (%) to observed dissimilarities between the two species (SIMPER).

vs. heterospecific, P = 1; conspecific vs. control, P<0.0001; heterospecific vs. control, P<0.001; Figure 3G). Copulation attempts were performed equally often toward dummies applied with CHCs from con- and heterospecific females ($2 \times 2\ \chi^2$ test: $\chi^2 = 0.2$, P = 0.63) and no copulation attempts were observed toward the solvent control. In contrast, *N. girault*i males spent more time on CHCs of heterospecific females than on both CHCs of conspecific females and solvent control (Kruskal-Wallis test: P<0.0001; Bonferroni-corrected Mann–Whitney U-test pairwise comparisons: conspecific vs. heterospecific, P = 0.02; conspecific vs. control, P = 0.83; heterospecific vs. control, P = 0.0001; Figure 3H). No copulation attempts were performed by *N. girault*i males in response to CHC fractions.

Analysis of the combined data from experiments 2–4 revealed significant differences in the general response of the two species. Males of *N. vitripennis* spent more time on dummies and attempted to copulate more often than males of *N. girault*i (glm, time mounted: $F_{1,355} = 122.7$; logistic regression model, copulation attempts: $\chi^2 = 95.1$, both P<0.0001; Table S4). In addition, the response of both species decreased with lower information content available (from dead females over complete body extracts to the CHC fraction alone; glm, time mounted: $F_{1,352} = 173.8$; logistic regression model, copulation attempts: $\chi^2 = 39.4$, both P<0.0001), and this effect on copulation attempts was different between the two species (logistic regression model, species*information content: $\chi^2 = 6.1$, P = 0.01).

Chemical composition of female-derived cuticular lipids

Females of *N. vitripennis* and *N. girault*i differed in the composition of their cuticular lipid profiles (PERMANOVA: Pseudo-$F_{1,18} = 26.8$, P<0.001; Figure 4; see Table 1 for a list of all identified compounds). The differences did not result from differences in the amounts of single components but were rather based on the combined effects of many components taken together. Almost two-thirds of the analyzed peaks were necessary to explain 90% of the observed differences between the two species and no single peak contributed more than 16.5% to these differences (SIMPER; Table 1). No differences were observed in the total amount of cuticular lipids between the species (*N. girault*i: mean ± SD = 730 ± 210 ng, *N. vitripennis*: 680 ± 180 ng; Mann–Whitney U-test: P = 0.62).

Nevertheless, differences were found in the branching patterns of CHCs. N-alkanes and monomethylalkanes were more abundant in *N. vitripennis* (Mann–Whitney U-test: $P<0.0001$ for both classes of alkanes), whereas multiply branched CHCs were more abundant in *N. giraulti* (Mann–Whitney U-test: dimethylalkanes: $P<0.01$; tri- and tetramethylalkanes: both $P<0.0001$). The only more polar lipid identified in the analyzed extracts was cholesterol.

Discussion

Both, *N. vitripennis* and *N. giraulti* males readily courted and successfully mated with conspecific females. In contrast to Buellesbach et al. (2013), we were able to show that chemical messengers are involved in the recognition of females in both *N. vitripennis* and *N. giraulti*. In addition, in both species, the intensity of the response decreased with decreasing information content of the chemical stimuli applied. In *N. vitripennis*, CHCs were sufficient for the recognition of con- and heterospecific females. In contrast, *N. giraulti* males depended on complete cuticular lipid extracts to recognize conspecific females, the CHC fraction alone was not sufficient.

Possible candidates used in the recognition of conspecific females by *N. giraulti* males are more polar lipids (Buckner, 1993), such as alcohols, aldehydes, ketones, wax esters, and non-volatile fatty acid derivatives (NFADs) such as TAGs. TAGs have been found to play an essential role in mate recognition in the parasitoid wasp *Lariophagus distinguendus* (Förster) (Kühbandner et al., 2012), and act as a brood pheromone in drone brood of the honey bee *Apis mellifera* L. (Koeniger & Veith, 1983). As they are not detected by standard GC-MS methods without transesterification into fatty acid methyl esters (Kühbandner & Ruther, 2015), NFADs were not detectable in the chemical analysis of cuticular lipids in this study.

Males of *N. vitripennis* recognize females by means of sex specific CHCs (Steiner et al., 2006). In accordance with the position of *N. vitripennis* at the basis of the phylogenetic tree (Campbell et al., 1993; Werren, 2010), recognition by CHCs seems to represent the ancestral state of mate recognition in this genus. If this is indeed the case, a shift to other chemical messengers must have happened in *N. giraulti*. Why this shift has happened remains unclear. Females of *N. giraulti* usually mate before emergence from the host, with reports of WHM rates ranging from 64 to 100% (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). Due to the confined space inside the host, chemical stimuli might be generally less important in mate recognition in this species. In our study, *N. giraulti* males showed generally weaker responses to the chemical stimuli presented than males of *N. vitripennis*. Males of *N. giraulti* spent less time mounted on extract-coated dummies and never tried to copulate with them. Decreased stabilizing selection on the fidelity of the chemical signal could thus have led to a drift in the composition of CHCs in *N. giraulti* females. Irrespective of the intensity of the response, males of *N. giraulti* did not discriminate between con- and heterospecific dead female dummies as well as between con- and heterospecific whole body extracts. These results are in accordance with Ruther et al. (2014), but contradict results from Giesbers et al. (2013), where males showed a preference for *N. vitripennis* female dummies over conspecific ones. This is all the more surprising, as both of these studies were based on the same inbred strain, namely NGVA 2. However, differences in data acquisition and long-time laboratory rearing of separated populations might account for the different results. Nevertheless, in bioassays with living couples we showed, that *N. giraulti* males engage in courtship faster when the presented female is conspecific, indicating that they are able to discriminate between con- and heterospecific mating partners and that they prefer conspecific ones. It seems therefore that *N. giraulti* males use other modes of communication, e.g., tactile cues or female movements, in addition to chemical messengers to recognize and discriminate between females. In contrast to our study, Buellesbach et al. (2014) found no discrimination of *N. giraulti* males against *N. vitripennis* females. However, they investigated only male mate rejection rates, possibly overlooking more subtle indicators of mate discrimination such as time effects in courtship and copulation.

In contrast to *N. giraulti*, males of *N. vitripennis* did not discriminate between con- and heterospecific females, and they readily responded to the CHC fraction of con- as well as heterospecific female extracts. These results are in accordance with earlier studies (Giesbers et al., 2013; Buellesbach et al., 2014). As males of *N. vitripennis* showed generally stronger responses to chemical stimuli, are faster in mounting and starting courtship (van den Assem & Werren, 1994; van den Assem & Beukeboom, 2004), and are more aggressive concerning other behaviors linked to mating (aggressive male-male interactions: Leonard & Boake, 2006; sneaking behavior: van den Assem & Beukeboom, 2004; Giesbers et al., 2013), they might be in general less sensitive to small changes in the composition of CHCs on the females' cuticle. Bioassays investigating the response of *N. vitripennis* males toward experimentally manipulated CHCs with decreased or increased relative amounts of individual substances could give valuable insights here (Kühbandner et al., 2013).

Nevertheless, the question remains why *N. giraulti* males recognize the CHC fraction of *N. vitripennis* female

extracts. If there has indeed been a shift in the composition of CHCs in *N. giraulti* females, one possibility is that the response toward CHCs of *N. vitripennis* merely persisted when the CHC composition in *N. giraulti* females changed. Another possibility is that *N. giraulti* males gain fitness benefits from engaging in courtship and copulation with heterospecific females. After emergence, males of *N. vitripennis* mark the substrate with a pheromone which is highly attractive to females (Ruther et al., 2007, 2008). During courtship, another pheromone is applied to the female antennae, leading to a behavioral switch as a result of which females cease to react to the abdominal sex pheromone (Ruther et al., 2010; Ruther & Hammerl, 2014). This switch is also triggered when the female is courted by a male of *N. giraulti* (Ruther et al., 2014). In addition, females of *Nasonia* usually consent to mating only once during their life time (Grillenberger et al., 2008). Females of *N. vitripennis* that have mated a heterospecific male are therefore likely to refuse mating again and might leave the host patch before having been inseminated by a conspecific. Due to cytoplasmic incompatibility, these females would then be unable to produce female offspring (Bordenstein et al., 2003). Clearly, an *N. giraulti* male having prevented an *N. vitripennis* female from mating a conspecific would only gain fitness benefits, if the probability that this female is going to oviposit on the same host patch as a conspecific female inseminated by the same male is considerably high. However, before such a scenario can be claimed, the proposed fitness benefits need to be demonstrated under field conditions.

Like *N. vitripennis*, females of *N. giraulti* discriminated against heterospecific males. In addition, we confirm that in *N. vitripennis* older females are less strict than younger females, and show that this age effect is also present in *N. giraulti* females. Decreased choosiness in older females is probably responsible for the high heterospecific mating rates in *N. giraulti* found in Giesbers et al. (2013). However, in Buellesbach et al. (2014) 2- to 3-day-old *N. giraulti* females discriminated significantly against *N. vitripennis* males suggesting strain-related variability in this feature. Nevertheless, our results show that female age has the potential to change results from mating trials profoundly, and is likely to be of importance in the other two species of *Nasonia* as well.

In conclusion, female-derived chemical stimuli are used by males of both species to recognize mating partners, although to a different degree. Our results stress the importance to control for age in mating trials with *Nasonia* wasps. In addition, they emphasize that a broad range of chemical substance classes need to be considered when investigating chemical communication in insects, even if the investigated species are closely related.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Results of the generalized linear model (with logit link function and pseudo-binomial error structure) calculated on the proportion of time males of *Nasonia vitripennis* and *N. giraulti* spent on post-copulatory courtship in bioassays with live con- and heterospecific couples. Species (*N. vitripennis*, *N. giraulti*) and type of pairing (conspecific, heterospecific) were included in the model. Factors and interactions not contributing significantly to the fit of the model were dropped using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).

Table S2. Results of (A) the logistic regression model calculated on the number of copulations of *Nasonia vitripennis* and *N. giraulti* females, and (B) the glm (with logit link function and pseudo-binomial error structure) calculated on the proportion of time males had to spend on head-nodding behavior prior to copulation, in bioassays with live con- and heterospecific couples. Species (*N. vitripennis*, *N. giraulti*), type of pairing (conspecific, heterospecific), and age (0-, 2-days old) were included in the model. Factors and interactions not contributing significantly to the fit of the model were dropped by a stepwise reduction in the model using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).

Table S3. Results of the generalized linear models calculated on the combined data from experiments 2–4 (see Material and methods section for description), based on separate analysis of (A) the proportion of time males of *Nasonia vitripennis* and *N. giraulti* spent mounted on dummies (glm with logit link function and pseudo-binomial error structure) and (B) the number of copulation attempts (logistic regression model). Species (*N. vitripennis*, *N. giraulti*), type of pairing (conspecific, heterospecific, solvent control), and information content [exp. 2: dead females (single confrontations); exp. 3: cuticular lipids; exp. 4: cuticular hydrocarbons fractions] were included as factors. Factors and interactions not contributing significantly to the fit of the model were dropped by a stepwise reduction in the model using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).