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Cuticular hydrocarbon divergence in the jewel wasp *Nasonia*: evolutionary shifts in chemical communication channels?

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sex pheromones;
signal receiver co-evolution;
speciation;
species discrimination.

Abstract

The evolution and maintenance of intraspecific communication channels constitute a key feature of chemical signalling and sexual communication. However, how divergent chemical communication channels evolve while maintaining their integrity for both sender and receiver is poorly understood. In this study, we compare male and female cuticular hydrocarbon (CHC) profiles in the jewel wasp genus *Nasonia*, analyse their chemical divergence and investigate their role as species-specific sexual signalling cues. Males and females of all four *Nasonia* species showed unique, nonoverlapping CHC profiles unambiguously separating them. Surprisingly, male and female phylogenies based on the chemical distances between their CHC profiles differed dramatically, where only male CHC divergence parallels the molecular phylogeny of *Nasonia*. In particular, *N. giraulti* female CHC profiles were the most divergent from all other species and very different from its most closely related sibling species *N. oneida*. Furthermore, although our behavioural assays indicate that female CHC profiles can generally be perceived as sexual cues attracting males in *Nasonia*, this function has apparently been lost in the highly divergent female *N. giraulti* CHC profiles. Curiously, *N. giraulti* males are still attracted to heterospecific, but not to conspecific female CHC profiles. We suggest that this striking discrepancy has been caused by an extensive evolutionary shift in female *N. giraulti* CHC profiles, which are no longer used as conspecific recognition cues. Our study constitutes the first report of an apparent abandonment of a sexual recognition cue that the receiver did not adapt to.

Introduction

The delicate interactions between transmission and perception of sexual cues are of fundamental importance to the evolution of intraspecific sexual communication (Endler, 1992). Stabilizing selection has been postulated as a possible mechanism to maintain established intraspecific communication channels (i.e. exclusive modalities of communication in one species), implying strong selection pressure against any disruptive shifts from both the signalling and the perception system (Paterson, 1980; Baker, 1989). However, concerning chemical communication, stabilizing selection alone would fail to explain the huge diversity of signalling molecules and the apparent capability to perceive and discriminate between them, which has been documented in decades of pheromone research (Symonds & Elgar, 2008).

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Moreover, if chemical communication channels are distinct between sympatric species, then highly specific coordination between sender and receiver in each channel has been postulated to contribute to prezygotic reproductive isolation (Löfstedt, 1993; Bargmann, 2006). Therefore, it is difficult to reconstruct how communication channels potentially diversified during the course of evolution, as the sender and the receiver must co-evolve for maintaining their integrity and functionality (Phelan, 1992).

Addressing this interesting evolutionary conundrum, some empirical evidence, primarily coming from research on moth sex pheromone communication, suggests the following scenario: in general, sexual selection results in more intense selection on males as receivers to track pheromonal profiles than on females as signallers to maintain a precise profile, as long as females are still successful at procuring mates (Phelan, 1992; Baker, 2002; Roelofs *et al.*, 2002). This would suggest a more narrowly tuned perception capability in the receiver contrasting a greater scope for alterations in the sexual cues of the sender (Phelan, 1992; Baker, 2002). Consequently, if senders change their chemical signals, receivers may experience increased selection pressure to adjust their perception, that is 'asymmetric tracking' (Phelan, 1997; Baker, 2002). Intriguingly, studies not only on sympatric moth species (Löfstedt *et al.*, 1991), but also on closely related bark beetles (Symonds & Elgar, 2004) and fruit flies (Ferveur, 2005) seem to support the assumption that their highly divergent pheromone compositions have evolved from major changes in the signal components of the senders (Symonds & Elgar, 2008). Furthermore, the risk of interspecific interference of chemical communication channels between sympatric species has been postulated to favour major changes in the compositions of sexual signals (Baker, 2002), allowing for increased mate discrimination and thus enhancing prezygotic reproductive isolation (Symonds & Elgar, 2008). However, few studies have addressed the issue of potential evolutionary changes in sexual communication channels in closely related species while considering both diverging signalling as well as perception capabilities. Thus, empirical evidence remains scarce and mostly limited to the examples mentioned above.

In this study, we analysed cuticular hydrocarbon (CHC) divergence between the four species of the jewel wasp genus *Nasonia* (Pteromalidae: Hymenoptera) and investigated their potential role in mate recognition. CHC are used as major chemical cues in sexual signalling (e.g. Cobb & Jallon, 1990; Simmons *et al.*, 2003; Oppelt & Heinze, 2009) and interspecific mate discrimination (e.g. Singer, 1998; Howard *et al.*, 2003; Peterson *et al.*, 2007) across a wide range of insect taxa. They are also commonly used as taxonomic characters (Carlson & Service, 1979; Howard *et al.*, 1988; Page *et al.*, 1997) that can, in some instances, help to reconstruct the evolutionary history of the respective taxa (Estradapena *et al.*, 1994; Mullen *et al.*, 2007; Martin *et al.*, 2008). In *Nasonia*, research on CHC profiles has thus far mostly focused on its most prominent and cosmopolitan member, *N. vitripennis*. Carlson *et al.* (1999) were the first to analyse CHC profiles in *N. vitripennis* and to report differences between the sexes. Later, it was established that female CHC profiles, in their entirety, function as sex pheromones in *N. vitripennis*, eliciting courtship behaviour in conspecific males (Steiner *et al.*, 2006). However, apart from *N. vitripennis*, three other *Nasonia* species have been described so far (Darling & Werren, 1990; Raychoudhury *et al.*, 2010). In contrast to the worldwide distribution of *N. vitripennis*, *N. longicornis* occurs exclusively in the western part of North America in sympatry with *N. vitripennis* (Darling & Werren, 1990), whereas *N. giraulti* and *N. oneida* both only occur in the eastern part of North America in sympatry with each other and *N. vitripennis* (Darling & Werren, 1990; Raychoudhury *et al.*, 2010). The only comparative CHC study between *Nasonia* species that has been conducted so far established that CHC profile differences could sufficiently separate *N. oneida* from its sibling species *N. giraulti* (Raychoudhury *et al.*, 2010).

With our CHC profile comparison encompassing all four *Nasonia* species, we explored whether CHC profiles are sufficiently distinct characters to discriminate all species and sexes. Furthermore, we analysed CHC divergence separately for males and females of all species and compared it with the known phylogenetic relationships in the *Nasonia* species complex. We did this to investigate whether the sexes differ in their respective CHC divergence and to elucidate the evolutionary mechanisms shaping CHC divergence for males and females.

We predicted to find congruence between CHC divergence and molecular phylogeny in males because so far no function of their CHC profiles in sexual communication could be documented, likely subjecting them to neutral evolution. In contrast, female CHC profiles have potentially undergone stabilizing or directional selection for their role in sexual communication, which we expected to detect in a divergence pattern aberrant from their molecular phylogeny. Finally, to complement our predictions, we conducted behavioural assays to determine whether the sex pheromone function documented in *N. vitripennis* females extends to the two most closely related species, *N. giraulti* and *N. oneida*, as well. In particular, we used the behavioural assays to test the sexual signalling function of conspecific vs. heterospecific female CHC profiles on *N. giraulti* and *N. oneida* males. Our goal was to trace changes in signalling and perception capabilities that have occurred during the evolution of CHC-based sexual communication channels in the *Nasonia* species complex and to assess whether

CHC-based sexual signalling is species-specific. Our results indicate that there has been a loss of the CHC signalling function in *N. giraulti* females, whereas *N. giraulti* males still retain the capability to recognize and react to female CHC profiles from other species. The significance and importance of these unexpected findings are discussed in the light of evolutionary mechanisms potentially responsible for shifts in chemical communication and corresponding changes in mating systems.

Materials and methods

Nasonia strains and rearing conditions

Natural populations of *Nasonia* are infected with the endosymbiont *Wolbachia* (Raychoudhury *et al.*, 2009). Thus, most laboratory lines of *Nasonia* have been antibiotically cured of their *Wolbachia* infection and have been in culture for many decades, without any apparent behavioural or fitness effects on the wasps due to the absence of *Wolbachia* (e.g. Beukeboom & van den Assem, 2001; Raychoudhury *et al.*, 2010). The standard laboratory strain for *N. vitripennis*, AsymCX, is the cured wild-type strain LBii (LabII), originally collected in Leiden (the Netherlands). Likewise, the standard laboratory strain for *N. giraulti*, RV2X(U), is an antibiotically cured version of the wild-type strain RV2, originally collected in Rochester, New York (Breeuwer & Werren, 1995). The *N. longicornis* strain IV7(U) is the cured IV7 strain originally collected in Utah, USA, and the *N. oneida* strain NONY11/36 has been collected in Brewerton, New York (Raychoudhury *et al.*, 2010). As an out-group for hierarchical clustering, we used a strain of the closely related pteromalid wasp species *Trichomalopsis sarcophagae*, ordered from Beneficial Insectary Inc.©, Redding, CA, USA, and originally collected in Alberta, Canada.

These are the same standard laboratory strains that have been used for the *Nasonia* genome sequencing (Werren *et al.*, 2010), and as we later compared molecular and CHC divergence, we attempted to keep strain-specific variation to a minimum by using the same strains for our study of chemical divergence. There is no evidence that endosymbionts have an effect on *Nasonia* CHC profiles (S. Vetter, J. Buellesbach & T. Schmitt, unpublished), and therefore, we utilized these endosymbiont-free laboratory lines as valid representative standard strains for our study of *Nasonia* CHC divergence.

Wasps were kept in plastic vials (97 mm height × 48 mm diameter) closed with foam plugs in an incubator (RUMED; Rubarth Apparate GMBH, Laatzen, Germany) under a constant temperature of 25 °C and a light/dark cycle of 16 : 8 h, resulting in a generation time of approximately 14 days. Pupae of the blowfly *Sarcophaga bullata* were used as hosts.

GC-MS analysis of the CHC profiles

Wasps were freeze-killed and stored separately by sex and species in glass vials at −20 °C after emergence from their hosts, as stable CHC profiles already appear in the late pupal stage of *Nasonia* (Carlson *et al.*, 1999) and also display sexual attractiveness on males even before eclosion, as documented in *N. vitripennis* (Steiner *et al.*, 2006). For CHC extraction, each wasp was placed for 10 min into 10 μL hexane. Extracts where then transferred to a fresh vial, concentrated by evaporating them with gaseous nitrogen to ~1 μL and subsequently injected into a gas chromatograph coupled with a mass selective detector (GC: 7890A; MS: 5975C; Agilent Technologies, Waldbronn, Germany) operating in electron impact ionization mode. The entire sample was injected in a split/splitless injector in the splitless mode with a temperature of 250 °C. Separation of compounds was performed on a fused silica capillary column (HP-5ms; Agilent Technologies) coated with a 0.25 μm (5%-phenyl)-methylpolysiloxane stationary phase with a temperature programme starting from 60 °C and increasing by 40 °C per min to 200 °C, followed by an increase of 5 °C per min to 320 °C. Peak area integration and calculation were performed using the data analysis software 'Enhanced Chemstation', G1701AA, version A.03.00 (Hewlett-Packard Company, Palo Alto, CA, USA). CHC compounds were identified according to their retention indices, diagnostic ions and mass spectra (Carlson *et al.*, 1998).

Profile comparison

Explorative data analyses and subsequent statistical analyses were performed with the program R, version 2.11.1 (R Development Core Team, 2010). To standardize the absolute peak area values, the normalization method of the function 'decostand' of the community ecology R package 'vegan' was used (Dixon, 2003), based on the following formula:

$$T_{x,y} = P_{x,y} / \sqrt{\sum P_y^2}$$

$T_{x,y}$ refers to the transformed peak area x of individual y, $P_{x,y}$ to the absolute peak area x of individual y and $\sum P_y^2$ to the squared sums of all absolute peak areas of individual y. This widely applied method for normalizing ecological data was chosen to make the peak areas comparable between our groups, to highlight the relative peak area differences and to correct for size-dependent variation.

Discriminant analysis

Discriminant analysis (DA) was performed with the R package 'MASS' (Venables & Ripley, 2002) to test whether the differences in relative amounts of the CHC

compounds sufficiently discriminate the eight predefined groups into the four species and two sexes. The numbers of individuals for females were 19, 17, 18 and 17 for *N. vitripennis*, *N. longicornis*, *N. giraulti* and *N. oneida*, respectively. Sample sizes for the males were 13, 11, 14 and 11; species order was same as above, amounting to an N_{total} of 124. To measure the quality of the DA, Wilk's λ, the correct assignments of individuals to their respective predefined groups, as well as the correct classification in leave-one-out cross-validation, were used. To visualize the data by plotting the first three discriminant functions simultaneously, the R package 'scatterplot3d' was used (Ligges & Maechler, 2003).

Hierarchical clustering

Agglomerative hierarchical cluster analysis ('unweighted pair-group method with arithmetic means', i.e. UPGMA) was performed with the R package 'ape' (Paradis *et al.*, 2004), based on the average chemical Manhattan distances between CHC extracts of the species-specific groups, performed separately for males and females. The formula for calculating Manhattan distances is as follows:

$$\sum |Y_j - Y_k|$$

The actual difference between two data points, in this case Y_j and Y_k, is used based on the total amount of CHC variation between the species. This opposes Euclidian distance, where squared differences are used and which is thus strongly dominated by single large differences. It has been proposed that most ecologically meaningful dissimilarities are of the Manhattan type (Oksanen, 2009).

A Mantel test (Mantel, 1967) conducted with the R package 'ade4' (Dray & Dufour, 2007) compared the molecular distances based on the *Nasonia* phylogeny (genetic divergence of a mitochondrial gene, COI sequences retrieved from NCBI, pairwise *P*-distances calculated with MEGA 3.0 (The Biodesign Institute, Tempe, AZ, USA), see Werren *et al.*, 2010) with the average Manhattan CHC distances, separately for males and females. CHC profiles of *T. sarcophagae* were used as an out-group for both analyses ($N_{male} = 12$ and $N_{female} = 14$), like in the molecular phylogeny of *Nasonia* (Werren *et al.*, 2010). Mantel tests were performed five times in each case, with 9999 permutations for each single test. The average probability from the five tests is presented.

Behavioural assays

Behavioural observations were performed in a mating chamber constructed as described by Ruther *et al.* (2000). The chamber consists of two acrylic glass plates ($60 \times 20 \times 5$ mm) screwed together. The upper glass plate contains a hole with a diameter of 10 mm that served as observation site for the behavioural assays. For assessing mate acceptance behaviour of *N. oneida* and *N. giraulti* males, single freeze-killed female dummies were offered to single virgin males for 5 min, and it was recorded whether the males initiated a copulation attempt with them, that is accepted them as a mating partner. The average mate acceptance rate was compared between con- and heterospecific pairings (see Fig. 4a,c) and between differentially treated female dummies (see Fig. 4b,d). Twenty replicates were conducted for each pairing, amounting to 240 single trials in total. In the comparisons between con- and heterospecific pairings, we paired *N. oneida* (see Fig. 4a) and *N. giraulti* (see Fig. 4c) males with female dummies of the species with which they co-occur in sympatry (Darling & Werren, 1990; Grillenberger *et al.*, 2009; Raychoudhury *et al.*, 2010); hence, we included female dummies from *N. vitripennis* in these trials as well. In the comparisons between differentially treated female dummies, we attempted to 'clear' the CHC profile off the females by keeping them in 2 mL hexane overnight after freeze-killing them. This treatment effectively removed the whole CHC profile with minute to nondetectable amounts remaining on the cuticle (see Fig. S1 for confirmation). Furthermore, we attempted to reconstitute female CHC profiles on the 'cleared' female dummies by applying 5 μL of CHC extract (equals to one female equivalent) from either conspecific or heterospecific single females to their washed cuticle. This effectively restored the female CHC profiles on the dummies to a level not significantly different from untreated dummies (see Fig. S1). We then proceeded to compare the mate acceptance rate of *N. oneida* (see Fig. 4b) and *N. giraulti* (see Fig. 4d) males with the female dummies treated as described above. Individual female dummies were used only once for each single mating trial. Bonferroni-corrected Fisher's exact tests assessed significant differences in mate acceptance rates.

Results

Discriminant analysis (DA) and CHC identifications

To assess whether the CHC profiles sufficiently distinguish sex and species in *Nasonia*, a total of 124 individuals were classified into eight groups, constituting the four species with both respective sexes, for a discriminant analysis (DA), according to CHC profile differences (Fig. 1). All profiles were significantly differentiated according to sex and species (Wilk's $\lambda < 0.00001$, $\chi^2 = 2072.81$, $P < 0.00001$). The DA could correctly assign 100% of the individuals to their respective groups. Similarly, 99.2% of the individuals were correctly classified in cross-validation. Discriminant

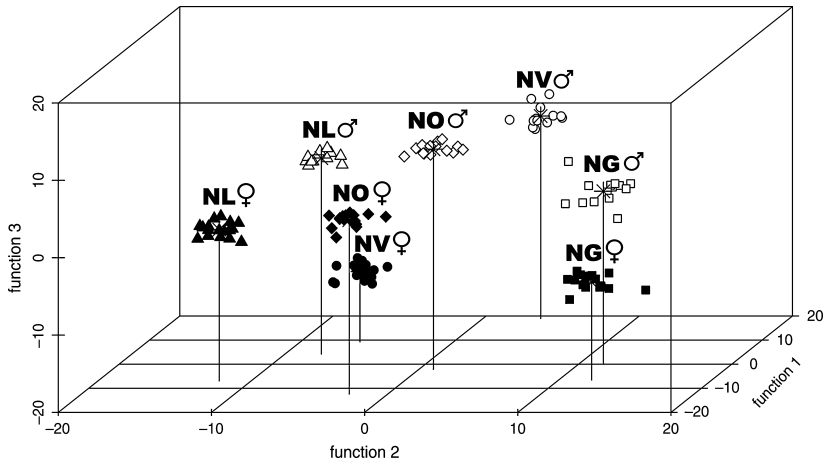

Fig. 1 Discriminant analysis (DA) of *Nasonia* cuticular hydrocarbon (CHC) profiles. Plot of the first three discriminant functions indicating the differences between 124 CHC profiles of males and females representative of the four *Nasonia* species simultaneously in three dimensions. Females and males are represented by closed and open symbols, respectively. Circles represent *N. vitripennis* (NV, N_f = 19, N_m = 13), squares *N. giraulti* (NG, N_f = 18, N_m = 14), triangles *N. longicornis* (NL, N_f = 17, N_m = 11), and diamonds *N. oneida* (NO, N_f = 17, N_m = 11). N_f refers to the sample size for females, N_m refers to the sample size for males.

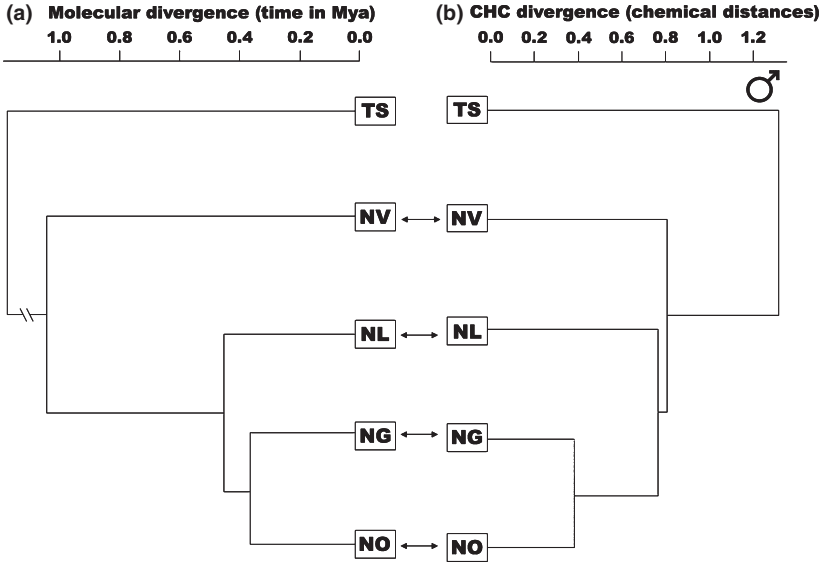

Fig. 2 Molecular phylogeny vs. male cuticular hydrocarbon (CHC) divergence. Comparison of *Nasonia* phylogeny based on mitochondrial DNA (a), and male chemical divergence of the four *Nasonia* species based on average Manhattan distances calculated between CHC extracts (b). NV = *N. vitripennis*; NG = *N. giraulti*; NO = *N. oneida*; NL = *N. longicornis*. Male CHC profiles of *Trichomalopsis sarcophagae* (TS) used as an out-group (N = 12). Other sample sizes for average chemical distances as in Fig. 1, molecular phylogeny adapted from Werren *et al.* (2010). Arrows indicate corresponding positions of males in CHC and molecular divergence.

function 1 accounted for 34.5%, function 2 for 27.1% and function 3 for 18% of the total variation, amounting to 79.6% of total variance explained by the first three functions. When the three functions were plotted simultaneously in a three-dimensional representation, each group was uniquely defined, and no visual overlap between any of the eight groups was found (Fig. 1). Detailed descriptions of the identified CHC compounds, relative CHC abundances and the distribution of CHC compound classes per sex and species are given in Table S1 and Fig. S2, respectively (see also description in Appendix S1).

Cluster analysis of CHC divergence and correlation with phylogeny

Hierarchical clustering of CHC divergence vs. phylogenetic divergence was performed separately for both sexes to investigate whether different sex-specific functions of the CHC profiles (mainly the function as sexual cues in females but not in males) are reflected in different tree topologies for males (Fig. 2) and females (Fig. 3). The molecular and chemical distance-based trees were rooted with the out-group *T. sarcophagae*, constituting the most distant branch in all cases. Figure 2b represents the chemical divergence of the male CHC profiles, and Table S2

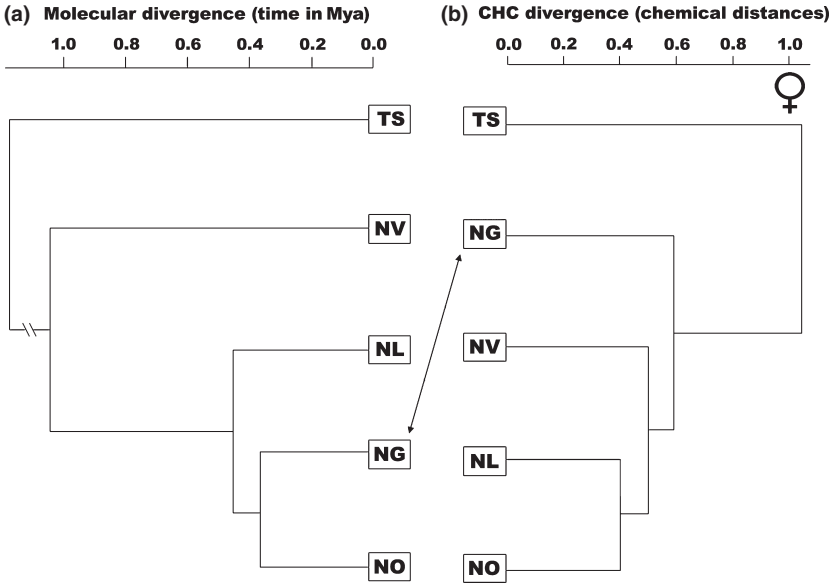

Fig. 3 Molecular phylogeny vs. female cuticular hydrocarbon (CHC) divergence. Comparison of *Nasonia* phylogeny based on mitochondrial DNA (a), and female chemical divergence of the *Nasonia* strains based on average Manhattan distances calculated between CHC extracts (b). Female CHC profiles of *Trichomalopsis sarcophagae* (TS) used as an out-group ($N = 14$), abbreviations and specifics as in Fig. 2. An arrow indicates the different positions of *N. giraulti* females contrasting CHC to molecular divergence.

shows the corresponding distance matrix. The two most recently diverged sibling species based on nuclear gene divergence, *N. giraulti* and *N. oneida* (Raychoudhury *et al.*, 2010), also cluster most closely together on the male chemical divergence tree, and *N. vitripennis* males constitute the most distant branch within the genus *Nasonia* for both nuclear gene and chemical divergence. This accurately reflects the molecular phylogeny of *Nasonia* (Figs 2 and 3a; Werren *et al.*, 2010). The Mantel test comparing the average CHC distances for each species cluster in the males with the molecular distances further confirms this concordance ($r = 0.519$, $P = 0.041$). This is strongly contrasted by the chemical divergence of female CHC profiles, displaying a markedly different pattern (Fig. 3b, see also Table S3 for the corresponding distance matrix). *Nasonia giraulti* females, in particular, show the most divergent CHC profiles in comparison with females of the other *Nasonia* species, which is also reflected in their isolated position in the discriminant analysis (see Fig. 1). A Mantel test found no significant correlation between the molecular and average chemical distances based on the CHC profiles of the females ($r = 0.287$, $P = 0.238$).

Behavioural assays

To determine the influence of female CHC profiles on male mating behaviour, we recorded copulation attempts of males from the two most closely related species, *N. giraulti* and *N. oneida*, against untreated con- and heterospecific female dummies (Fig. 4a,c) and against treated (i.e. CHC-'cleared' or CHC-'reconstituted') female dummies (Fig. 4b,d).

Nasonia oneida males showed a clear preference of conspecific over both heterospecific *N. giraulti* (Fisher's exact test, $P < 0.001$) and *N. vitripennis* (Fisher's exact test, $P < 0.001$) female dummies (Fig. 4a), both of which naturally occur in sympatry with *N. oneida* (Raychoudhury *et al.*, 2010). Conspecific female dummies with 'cleared' CHC profiles elicited no copulation attempts by *N. oneida* males. But when cleared dummies were reconstituted with conspecific female CHC extracts, *N. oneida* males attempted to copulate (Fisher's exact test, $P < 0.001$, Fig. 4b). Cleared *N. oneida* female dummies with reconstituted heterospecific *N. giraulti* female CHC extracts did not elicit copulation responses in conspecific males significantly more often than completely cleared *N. oneida* female dummies (Fisher's exact test, $P = 1$, Fig. 4b).

Nasonia giraulti males, on the other hand, did not show any copulation attempts on conspecific, but significantly more attempts on both heterospecific *N. vitripennis* (Fisher's exact test, $P < 0.001$) and *N. oneida* (Fisher's exact test, $P < 0.001$) female dummies (Fig. 4c). In addition, neither *N. giraulti* female dummies with cleared CHC profiles nor reconstituted with conspecific *N. giraulti* female CHC extracts elicited any copulation attempts from *N. giraulti* males (Fig. 4d). However, significantly more copulation attempts than in the latter two cases were elicited by cleared *N. giraulti* female dummies reconstituted with heterospecific *N. oneida* female CHC extract (Fig. 4d, Fisher's exact test, $P < 0.001$).

Discussion

CHC divergence and their role as species-specific sexual cues

In the present study, we investigated whether CHC profile can function as species-specific female sexual cues and explored how CHC divergence potentially evolved with respect to the genetic divergence of the *Nasonia* species complex. Our results showed unique CHC

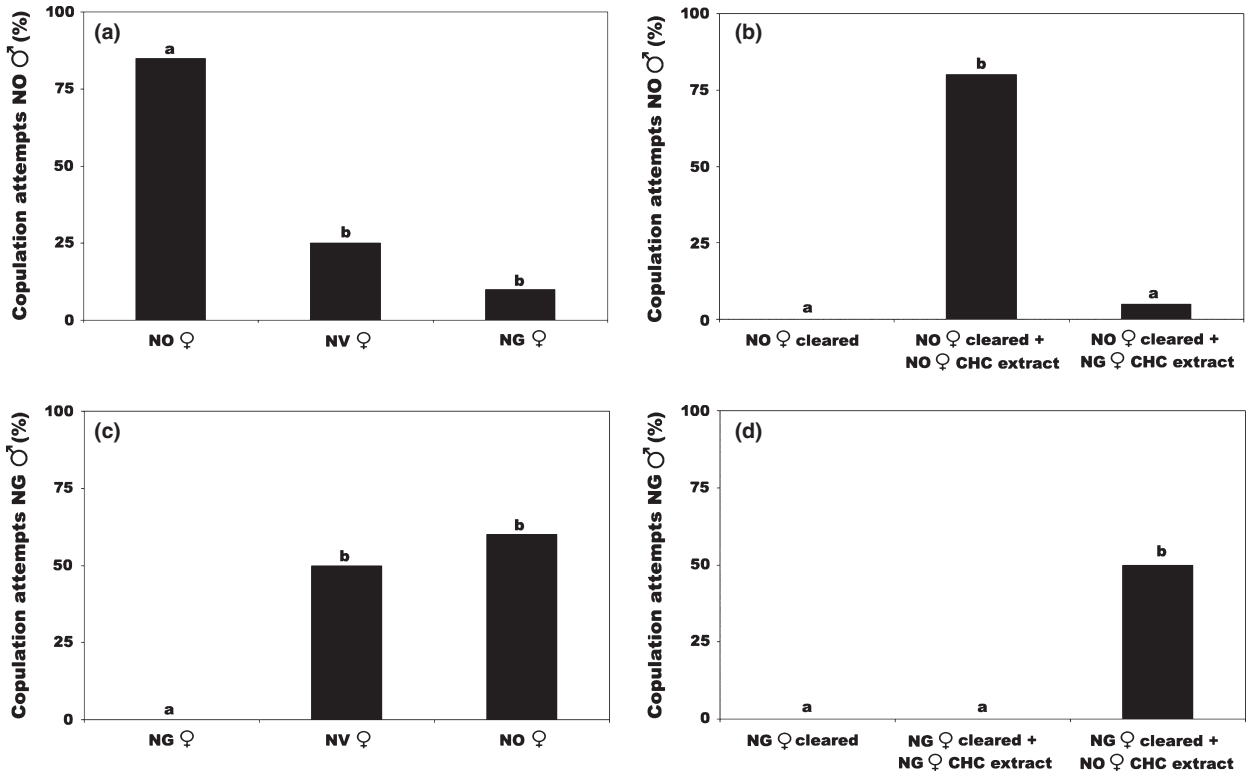

Fig. 4 Behavioural assays with *Nasonia oneida* and *N. giraulti* males on female dummies. (a) Percentage of copulation attempts of *N. oneida* males with conspecific and with heterospecific female dummies from the sympatric species *N. vitripennis* and *N. giraulti*, respectively. (b) Percentage of copulation attempts of *N. oneida* males with 'cleared' [cuticular hydrocarbon (CHC)-erased] conspecific female dummies, dummies reconstituted with conspecific female CHC extract and dummies reconstituted with heterospecific *N. giraulti* female CHC extract, respectively. (c) Percentage of copulation attempts of *N. giraulti* males with conspecific and with heterospecific *N. vitripennis* and *N. oneida* female dummies, respectively. (d) Percentage of copulation attempts of *N. giraulti* males with 'cleared' conspecific female dummies, dummies reconstituted with conspecific female CHC extract and dummies reconstituted with heterospecific *N. oneida* female CHC extract, respectively. Two hundred and forty assays were performed in total, 20 replicates per pairing, and different letters indicate highly significant ($P < 0.001$) absolute differences in number of copulation attempts based on Bonferroni-corrected Fisher's exact tests, independently tested in a–d.

profiles with no overlaps for all four *Nasonia* species and both sexes, respectively (Fig. 1), suggesting them to be both species- and sex-specific traits within this genus. CHC species specificity has been documented in a wide variety of insect taxa (Carlson & Service, 1980; Howard *et al.*, 1982; Carlson & Milstrey, 1991) and generally hints at their potential to play a role in prezygotic reproductive isolation. Sex-specificity has also been found to be a widely distributed, but not a universal characteristic of insect CHC profiles (Thomas & Simmons, 2008) and constitutes a prerequisite for CHC to function as cues in sexual communication. Thus, we established that CHC profiles in *Nasonia* bear all the necessary characteristics of species-specific sexual cues. Moreover, distinct CHC profiles in males and females of *T. sarcophagae*, a close relative of *Nasonia*, hint at an early evolution of sex-specific CHC differences potentially preceding the radiation of the *Nasonia* genus (Table S1 and Fig. S2). We tested single strains representative of the four *Nasonia* species as no strain-specific variation in *Nasonia* CHC profiles could be detected so far (Raychoudhury *et al.*, 2010, W. Diao & T. Schmitt, unpublished), supporting that CHC indeed constitute genetically fixed characters unique for each species and sex. However, minor CHC variation between different populations in one species cannot be ruled out completely and should be investigated in further studies.

As previously shown to function as female-specific sexual cues in *N. vitripennis* (Steiner *et al.*, 2006), we confirmed this role for CHC profiles in the present study for *N. oneida* (Fig. 4a–b). Rather unexpectedly, males of *N. oneida* sibling species, *N. giraulti*, appear to only perceive CHC profiles of heterospecific, but not of conspecific females as sexual cues (Fig. 4c–d). Our comparison of CHC divergence with the molecular phylogeny of *Nasonia*, performed separately for males and females, delivers further insights into this unexpected observation (Figs 2 and 3). Male CHC divergence of the four investigated *Nasonia* species shows concordance with

their genetic divergence (Fig. 2, $r = 0.927$, $P = 0.041$), whereas female CHC profile divergence does not (Fig. 3, $r = 0.353$, $P = 0.238$). CHC profiles of *N. giraulti* females deviate the most from the molecular divergence pattern and also constitute the most distant profile in the CHC divergence of all *Nasonia* females (Fig. 3b). As the same strains were used for our comparison of chemical with molecular divergence as in the *Nasonia* genome project, we kept the factor of strain-specific genetic variation to a minimum. Our results strongly hint at an evolutionary shift that apparently affected *N. giraulti* female CHC profiles so strongly that conspecific males no longer respond to them as mate recognition cues, although the males retain an 'ancestral' attraction to CHC profiles of females from their sibling species, confirmed for three separate *N. giraulti* strains (see below). However, future studies should investigate and confirm this behaviour in further populations.

Species-specific CHC-based communication and reproductive isolation

In natural populations, *N. giraulti* and *N. vitripennis* have been found microsympatrically, within bird nests in eastern North America, occasionally even sharing the same single host pupa (Darling & Werren, 1990; Grillenberger *et al.*, 2009). The sibling species *N. oneida* has only recently been discovered (Raychoudhury *et al.*, 2010). Although it is currently found in sympatry with *N. giraulti* and *N. vitripennis* (Grillenberger *et al.*, 2009; Raychoudhury *et al.*, 2010), it was not detected in eastern North American populations (or elsewhere) until after 2000, despite extensive sampling (J.H. Werren, unpublished). Concerning perception and discrimination of female CHC profile, which might contribute to prezygotic reproductive isolation between the three sympatric species, *N. oneida* males showed a clear preference of con- over heterospecific female CHC profile (Fig. 4a–b). *Nasonia giraulti* males, on the other hand, apparently retain the capability of detecting and reacting to an ancestral female CHC sexual cue still present in *N. vitripennis* as well as *N. oneida* females, but not in *N. giraulti* females (Fig. 4c–d). These findings suggest that intact female-specific CHC communication constitutes an ancestral state in *Nasonia* and that this trait has been lost in *N. giraulti*.

Interestingly, *N. oneida* and *N. giraulti* constitute the only *Nasonia* species pair where no post-zygotic reproductive isolation has been found under laboratory conditions (Raychoudhury *et al.*, 2010). In all other species pairings, different species-specific *Wolbachia* strains cause different degrees of cytoplasmatic incompatibility, which either greatly reduce or completely eliminate the occurrence of hybrids, thus acting as a strong post-zygotic reproductive barrier (Breeuwer & Werren, 1990; Bordenstein & Werren, 1998; Bordenstein *et al.*, 2001; Raychoudhury *et al.*, 2010). Although antibiotically treated and thus *Wolbachia*-free strains were used in our behavioural assays, we could exclude that *Wolbachia* affected the attractiveness of *N. giraulti* female CHC profiles, because assays with two uncured laboratory *N. giraulti* strains yielded the same results (i.e. no copulation attempts from males with freeze-killed female dummies in both cases, J. Buellesbach, F. Echinger & T. Schmitt, unpublished). Albeit *N. giraulti* and *N. oneida* are infected with the same *Wolbachia* strains and no other post-zygotic reproductive isolation mechanisms could be documented, they clearly constitute separate species with distinct genetic, behavioural and subtle morphological differences (Raychoudhury *et al.*, 2010; see also Figs 2 and 3a). Female mate rejection against heterospecific males has been shown to be exceptionally strong in *N. oneida* females (Raychoudhury *et al.*, 2010), but it is doubtful whether this is the only prezygotic isolation mechanism that keeps these two most closely related, not post-zygotically isolated *Nasonia* sibling species separated in nature.

Another important biological feature of *N. giraulti* is Within-host mating. Whereas most *Nasonia* species routinely mate following emergence from the host, *N. giraulti* shows a uniquely high propensity for mating within the host prior to emergence (Drapeau & Werren, 1999; Leonard & Boake, 2006), and it has already been hypothesized that Within-host mating evolved as a mechanism to escape hybridization with *N. vitripennis*, with which it commonly co-occurs within bird nests in eastern North America (Darling & Werren, 1990; Drapeau & Werren, 1999).

Abandonment of species-specific CHC-based communication in *N. giraulti*

What could have caused the major shift in *N. giraulti* female CHC profiles, apparently even inhibiting the capability of conspecific males to perceive them as sexual cues? We propose two alternatives to explain this dramatic shift, referred to as 'CHC evasion' and 'Within-host mating' models, respectively, differing in order and cause of evolutionary events.

The CHC evasion model proposes that diverging selection shifted female CHC profiles in *N. giraulti* to establish a species-specific CHC-based communication channel with no cross-attraction from heterospecific males, particularly from *N. oneida* where no post-zygotic reproductive isolation mechanisms against *N. giraulti* could be documented so far. As proposed by the asymmetric tracking hypothesis, if accompanied by an adaptation of the receiver to a shift in the sender, new communication channels can theoretically emerge, separating different species (Phelan, 1997; Baker, 2002). However, the proposed asymmetric tracking process apparently did not take place in *N. giraulti* males, with them not properly adapting to the shifted female CHC profiles as sexual cues. Interestingly, it has

been hypothesized that asymmetric tracking generally occurs quite rarely in the evolution of chemical communication systems (Roelofs *et al.*, 2002). Thus, other potential premating isolation mechanisms and sexual cues became vital in this species to distinguish confrom heterospecific females in sympatric populations. When not exclusively reliant on CHC signalling as in our specific experimental set-up, *N. giraulti* males still recognize alive conspecific females and attempt to court and mate with them (van den Assem & Werren, 1994; Drapeau & Werren, 1999; J. Buellesbach, C. Greim & T. Schmitt, unpublished), strongly suggesting that CHC-based sexual signalling has been replaced by other modes of communication in this species. These potentially include male-based chemical signalling (Niehuis *et al.*, 2013), behavioural and tactile cues (J. Buellesbach, C. Greim & T. Schmitt, unpublished) and acoustic cues based on sounds produced through wing fanning (W. Diao, unpublished). These observations support the hypothesis that the shift implied by the CHC deviation model was partially induced to avoid interspecific male interference, favouring switches to other modes of intraspecific communication, whereas *N. giraulti* males retained an ancestral attraction to heterospecific female CHC profiles. The successful procuring of mates by *N. giraulti* females via other modes of sexual communication could have facilitated the abandonment of the reliance on CHC-based sexual signalling in this species.

Conversely, in the Within-host mating model, high frequencies of Within-host mating first evolved in *N. giraulti* as a mechanism to avoid heterospecific matings (Drapeau & Werren, 1999; Leonard & Boake, 2006), leading to the relaxation of selection on female CHC profile as mate recognition cues in *N. giraulti* and subsequently to the complete loss of that function. A likely consequence of exclusively mating within the host is that the necessity of species-specific mate recognition through CHC profiles is reduced, given the close proximity of mating pairs, the relatively scarce occurrence of multiply infected hosts under natural conditions (J.H. Werren, unpublished) and the fact that all other species do not routinely mate within the host (Drapeau & Werren, 1999; Leonard & Boake, 2006; M.C.W.G. Giesbers & L.W. Beukeboom unpublished). Furthermore, the strongly female-biased sex ratios produced by *N. giraulti* (Drapeau & Werren, 1999) imply that males almost exclusively encounter females within the host under natural conditions, potentially further reducing the need for CHC-based sexual signalling. Thus, according to the Within-host mating model, the decrease in the reliance on CHC profiles as sexual cues due to the prominence of Within-host mating in *N. giraulti* released female CHC profiles from stabilizing selection to act as species-specific sexual cues, leading to their aberrant profiles.

Both the CHC evasion and the Within-host mating model represent hypotheses on how the apparent abandonment of CHC-based sexual communication in *N. giraulti* has potentially occurred in the course of evolution. Future studies might be able to delve further into the hypothetical framework, advancing our understanding of these unexpected observations and possibly even shedding light on which hypothesis more likely reflects the evolutionary cause of events, a distinction that currently cannot be made.

Evolution of CHC divergence in males and females

To the best of our knowledge, the present study is the first to show substantial differences between males and females comparing chemical and genetic divergence within the same taxon (Figs 2 and 3). Previous studies comparing CHC divergence with molecular phylogenies have yielded inconsistent results. For instance, in the pine engraver beetle genus *Ips* (Page *et al.*, 1997), the *Drosophila mojavensis* (Etges & Jackson, 2001) and *D. buzzatii* (Oliveira *et al.*, 2011) species clusters and the termite genus *Reticulitermes* (Page *et al.*, 2002), CHC profile divergence matched the phylogenetic relationships. In other taxa, for example, in cockroaches of the *Cryptocercus punctulatus* species complex (Everaerts *et al.*, 2008) and the fungus-growing termite genus *Macrotermes* (Marten *et al.*, 2009), no concordance was found between CHC divergence and molecular phylogeny. Hence, whether or not CHC divergence is congruent with species phylogeny appears to be highly dependent on the studied taxa. This might also indicate that selection on CHC profile for different functions can override their phylogenetic information. However, none of the previous studies attempted to differentiate male and female CHC divergence and to independently correlate it with the respective phylogenies. Our results suggest that different evolutionary forces have been shaping CHC divergence in males and females of the *Nasonia* species complex. Selection for a function as a female-specific sex pheromone in *N. vitripennis* (Steiner *et al.*, 2006) and *N. oneida* (Fig. 4a–b) hints at evolutionary constraints restricting CHC divergence in females (apart from *N. giraulti* as demonstrated by our study), but not in males.

Male CHC divergence, in turn, has likely evolved neutrally as males participate in the communication channels as the receivers with no apparent reliance on their CHC profiles as sexual cues. CHC divergence in males tracks the phylogenetic divergence much more closely than in females. Thus, different evolutionary mechanisms potentially explain the distinct CHC divergence patterns separating the sexes of the four *Nasonia* species.

Conclusions

We have shown substantially different CHC profiles for males and females of the four *Nasonia* species and signifi-

cant differences in male and female CHC divergence patterns. The most likely scenario to explain the functional separation of male from female CHC profiles is the role of the latter as sexual cues. If intact CHC-based sexual communication constitutes an ancestral state in *Nasonia*, as our data suggest, then *N. giraulti* presents a particularly interesting and unique case of an abandonment of this species-specific communication modality. We suggest an evolutionary shift of *N. giraulti* female CHC profiles, either induced to establish an exclusive species-specific CHC-based communication channel ('CHC deviation' model) or as a gradual loss-of-function of CHC profiles as sexual cues due to the prominence of Within-host mating in *N. giraulti* ('Within-host mating' model). Curiously, *N. giraulti* males retain an apparently ancestral attraction to heterospecific female CHC profiles. Our study provides a promising basis for further investigating the mechanisms governing CHC-based mate assessment and species discrimination. Recent studies in other insect taxa point at a much more complicated scenario for CHC-mediated sexual signalling than previously assumed, hinting at the biological significance of minute differences in CHC compound ratios for differential signalling as well as perception capabilities (Ibeas *et al.*, 2009; Thomas & Simmons, 2009; Everaerts *et al.*, 2010). These studies indicate the need for a suitable model organism for investigating CHC profile variation and its role in species-specific communication channels. Complementing our results in future studies with its numerous advantages, for example sequenced genomes and male haploidy (Werren & Loehlin, 2009; Niehuis *et al.*, 2010; Werren *et al.*, 2010), *Nasonia* might prove to be a particularly well-suited model system for further investigating the genetic basis (Niehuis *et al.*, 2011) and the exact mechanisms that underlie CHC-based sexual communication and its evolution.

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References

van den Assem, J. & Werren, J.H. 1994. A comparison of the courtship and mating behavior of three species of *Nasonia* (Hymenoptera: Pteromalidae). *J. Insect Behav.* **7**: 53–66.

Baker, T.C. 1989. Sex-pheromone communication in the Lepidoptera: new research progress. *Experientia* **45**: 248–262.

Baker, T.C. 2002. Mechanism for saltational shifts in pheromone communication systems. *Proc. Natl. Acad. Sci. USA* **99**: 13368–13370.

Bargmann, C.I. 2006. Comparative chemosensation from receptors to ecology. *Nature* **444**: 295–301.

Beukeboom, L.W. & van den Assem, J. 2001. Courtship and mating behaviour of interspecific *Nasonia* hybrids (Hymenoptera, Pteromalidae): a grandfather effect. *Behav. Genet.* **31**: 167–176.

Bordenstein, S.R. & Werren, J.H. 1998. Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics* **148**: 1833–1844.

Bordenstein, S.R., O'Hara, F.P. & Werren, J.H. 2001. *Wolbachia* induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**: 707–710.

Breeuwer, J.A.J. & Werren, J.H. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346**: 558–560.

Breeuwer, J.A.J. & Werren, J.H. 1995. Hybrid breakdown between two haplodiploid species: the role of nuclear and cytoplasmic genes. *Evolution* **49**: 705–717.

Carlson, D.A. & Milstrey, S.K. 1991. Alkanes of 4 related moth species, *Helicoverpa* and *Heliothis*. *Arch. Insect Biochem. Physiol.* **16**: 165–175.

Carlson, D.A. & Service, M.W. 1979. Differentiation between species of the *Anopheles gambiae* giles complex (Diptera, Culicidae) by analysis of cuticular hydrocarbons. *Ann. Trop. Med. Parasitol.* **73**: 589–592.

Carlson, D.A. & Service, M.W. 1980. Identification of mosquitos of *Anopheles gambiae* species complex A and complex B by analysis of cuticular components. *Science* **207**: 1089–1091.

Carlson, D.A., Bernier, U.R. & Sutton, B.D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* **24**: 1845–1865.

Carlson, D.A., Geden, C.J. & Bernier, U.R. 1999. Identification of pupal exuviae of *Nasonia vitripennis* and *Muscidifurax raptorellus* parasitoids using cuticular hydrocarbons. *Biol. Control* **15**: 97–105.

Cobb, M. & Jallon, J.M. 1990. Pheromones, mate recognition and courtship stimulation in the *Drosophila melanogaster* species subgroup. *Anim. Behav.* **39**: 1058–1067.

Darling, D.C. & Werren, J.H. 1990. Biosystematics of *Nasonia* (Hymenoptera: Pteromalidae): two new species reared from birds' nests in North America. *Ann. Entomol. Soc. Am.* **83**: 352–370.

Dixon, P. 2003. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **14**: 927–930.

Drapeau, M.D. & Werren, J.H. 1999. Differences in mating behaviour and sex ratio between three sibling species of *Nasonia*. *Evol. Ecol. Res.* **1**: 223–234.

Dray, S. & Dufour, A.B. 2007. The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* **22**: 1–20.

Endler, J.A. 1992. Signals, signal conditions, and the direction of evolution. *Am. Nat.* **139**: 125–153.

Estradapena, A., Castella, J. & Moreno, J.A. 1994. Using cuticular hydrocarbon composition to elucidate phylogenies in tick populations (Acari, Ixodidae). *Acta Trop.* **58**: 51–71.

Etges, W.J. & Jackson, L.L. 2001. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. VI. Epicuticular hydrocarbon variation in

Drosophila mojavensis cluster species. *J. Chem. Ecol.* **27**: 2125–2149.

Everaerts, C., Maekawa, K., Farine, J.P., Shimada, K., Luykx, P., Brossut, R. *et al.* 2008. The *Cryptocercus punctulatus* species complex (Dictyoptera: Cryptocercidae) in the eastern United States: comparison of cuticular hydrocarbons, chromosome number, and DNA sequences. *Mol. Phylogenet. Evol.* **47**: 950–959.

Everaerts, C., Farine, J.P., Cobb, M. & Ferveur, J.F. 2010. *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *PLoS ONE* **5**: e9607.

Ferveur, J.-F. 2005. Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. *Behav. Genet.* **35**: 279–295.

Grillenberger, B.K., van de Zande, L., Bijlsma, R., Gadau, J. & Beukeboom, L.W. 2009. Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera). *J. Evol. Biol.* **22**: 460–470.

Howard, R.W., McDaniel, C.A., Nelson, D.R., Blomquist, G.J., Gelbaum, L.T. & Zalkow, L.H. 1982. Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species-recognition and caste-recognition cues. *J. Chem. Ecol.* **8**: 1227–1239.

Howard, R.W., Thorne, B.L., Levings, S.C. & McDaniel, C.A. 1988. Cuticular hydrocarbons as chemotaxonomic characters for *Nasutitermes corniger* (Motschulsky) and *Nasutitermes ephratae* (Holmgren) (Isoptera, Termitidae). *Ann. Entomol. Soc. Am.* **81**: 395–399.

Howard, R.W., Jackson, L.L., Banse, H. & Blows, M.W. 2003. Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: identification and role in mate choice in *D. serrata*. *J. Chem. Ecol.* **29**: 961–976.

Ibeas, F., Gemeno, C., Diez, J.J. & Pajares, J.A. 2009. Female recognition and sexual dimorphism of cuticular hydrocarbons in *Monochamus galloprovincialis* (Coleoptera: Cerambycidae). *Ann. Entomol. Soc. Am.* **102**: 317–325.

Leonard, J.E. & Boake, C.R.B. 2006. Site-dependent aggression and mating behaviour in three species of *Nasonia* (Hymenoptera: Pteromalidae). *Anim. Behav.* **71**: 641–647.

Ligges, U. & Maechler, M. 2003. Scatterplot3d – An R package for visualizing multivariate data. *J. Stat. Softw.* **8**: 1–20.

Löfstedt, C. 1993. Moth pheromone genetics and evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **340**: 167–177.

Löfstedt, C., Herrebout, W.M. & Menken, S.B.J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* **2**: 20–28.

Mantel, N. 1967. Detection of disease clustering and a generalized regression approach. *Cancer Res.* **27**: 209–220.

Marten, A., Kaib, M. & Brandl, R. 2009. Cuticular hydrocarbon phenotypes do not indicate cryptic species in fungus-growing termites (Isoptera: Macrotermitinae). *J. Chem. Ecol.* **35**: 572–579.

Martin, S.J., Helantera, H. & Drijfhout, F.P. 2008. Evolution of species-specific cuticular hydrocarbon patterns in Formica ants. *Biol. J. Linn. Soc.* **95**: 131–140.

Mullen, S.P., Mendelson, T.C., Schal, C. & Shaw, K.L. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: Laupala). *Evolution* **61**: 223–231.

Niehuis, O., Gibson, J.D., Rosenberg, M.S., Pannebakker, B.A., Koevoets, T., Judson, A.K. *et al.* 2010. Recombination and its impact on the genome of the haplodiploid parasitoid wasp *Nasonia*. *PLoS ONE* **5**: e8597.

Niehuis, O., Buellesbach, J., Judson, A.K., Schmitt, T. & Gadau, J. 2011. Genetics of cuticular hydrocarbon differences between males of the parasitoid wasps *Nasonia giraulti* and *Nasonia vitripennis*. *Heredity* **107**: 61–70.

Niehuis, O., Buellesbach, J., Gibson, J.D., Pothmann, D., Hanner, C., Mutti, N.S. *et al.* 2013. Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* **494**: 345–348.

Oksanen, J. 2009. Multivariate analysis of ecological communities in R: vegan tutorial. http://cc.oulu.fi/~jarioksa/opetus/metodi/vegantutor.pdf

Oliveira, C.C., Manfrin, M.H., Sene, F. de M., Jackson, L.L. & Etges, W.J. 2011. Variations on a theme: diversification of cuticular hydrocarbons in a clade of cactophilic Drosophila. *BMC Evol. Biol.* **11**: 179.

Oppelt, A. & Heinze, J. 2009. Mating is associated with immediate changes of the hydrocarbon profile of *Leptothorax gredleri* ant queens. *J. Insect Physiol.* **55**: 624–628.

Page, M., Nelson, L.J., Blomquist, G.J. & Seybold, S.J. 1997. Cuticular hydrocarbons as chemotaxonomic characters of pine engraver beetles (*Ips* spp.) in the *grandicollis* subgeneric group. *J. Chem. Ecol.* **23**: 1053–1099.

Page, M., Nelson, L.J., Forschler, B.T. & Haverty, M.I. 2002. Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **131**: 305–324.

Paradis, E., Claude, J. & Strimmer, K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**: 289–290.

Paterson, H.E. 1980. A comment on mate recognition systems. *Evolution* **34**: 330–331.

Peterson, M.A., Dobler, S., Larson, E.L., Juarez, D., Schlarbaum, T., Monsen, K.J. *et al.* 2007. Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). *Chemoecology* **17**: 87–96.

Phelan, P.L. 1992. Evolution of sex pheromones and the role of asymmetric tracking. In: *Insect Chemical Ecology* (B.D. Roitberg & M.B. Isman, eds), pp. 265–314. Chapman & Hall, New York, NY.

Phelan, P.L. 1997. Genetics and phylogenetics in the evolution of sex pheromones. In: *Insect Pheromone Research* (R.T. Cadré & A.K. Minks, eds), pp. 563–579. Chapman & Hall, New York, NY.

R Development Core Team 2010. R: a language and environment for statistical computing. R foundation for statistical computing, http://www.R-project.org, Vienna, Austria.

Raychoudhury, R., Baldo, L., Oliveira, D.C.S.G. & Werren, J.H. 2009. Modes of acquisition of Wolbachia: horizontal transfer, hybrid introgression, and codivergence in the Nasonia species complex. *Evolution* **63**: 165–183.

Raychoudhury, R., Desjardins, C.A., Buellesbach, J., Loehlin, D.W., Grillenberger, B.K., Beukeboom, L.W. *et al.* 2010. Behavioral and genetic characteristics of a new species of *Nasonia*. *Heredity* **104**: 278–288.

Roelofs, W.L., Liu, W.T., Hao, G.X., Jiao, H.M., Rooney, A.P. & Linn, C.E. 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci. USA* **99**: 13621–13626.

Ruther, J., Homann, M. & Steidle, J.L.M. 2000. Female-derived sex pheromone mediates courtship behaviour in the

parasitoid *Lariophagus distinguendus*. *Entomol. Exp. Appl.* **96**: 265–274.
Simmons, L.W., Alcock, J. & Reeder, A. 2003. The role of cuticular hydrocarbons in male attraction and repulsion by female Dawson's burrowing bee, *Amegilla dawsoni*. *Anim. Behav.* **66**: 677–685.
Singer, T.L. 1998. Roles of hydrocarbons in the recognition systems of insects. *Am. Zool.* **38**: 394–405.
Steiner, S., Hermann, N. & Ruther, J. 2006. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J. Chem. Ecol.* **32**: 1687–1702.
Symonds, M.R.E. & Elgar, M.A. 2004. The mode of pheromone evolution: evidence from bark beetles. *Proc. R. Soc. Lond. B Biol. Sci.* **271**: 839–846.
Symonds, M.R.E. & Elgar, M.A. 2008. The evolution of pheromone diversity. *Trends Ecol. Evol.* **23**: 220–228.
Thomas, M.L. & Simmons, L.W. 2008. Sexual dimorphism in cuticular hydrocarbons of the Australian field cricket *Teleogryllus oceanicus* (Orthoptera: Gryllidae). *J. Insect Physiol.* **54**: 1081–1089.
Thomas, M.L. & Simmons, L.W. 2009. Sexual selection on cuticular hydrocarbons in the Australian field cricket, *Teleogryllus oceanicus*. *BMC Evol. Biol.* **9**: 162–174.
Venables, W.N. & Ripley, B.D. 2002. *Modern Applied Statistics with S*, 4th edn. Springer, New York, NY.
Werren, J.H. & Loehlin, D.W. 2009. The parasitoid wasp *Nasonia*: an emerging model system with haploid male genetics. *Cold Spring Harb. Protoc.* doi: 10.1101/pdb.emo134.
Werren, J.H., Richards, S., Desjardins, C.A., Niehuis, O., Gadau, J., Colbourne, J.K. *et al.* 2010. Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* **327**: 343–348.

Supporting information

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

Appendix S1 Species- and sex-specific distribution of CHC compounds.

Figure S1 Boxplots comparing the means of the total CHC amounts of female dummies that underwent the same treatments as for the behavioral assays (see Fig. 4 in main text).

Figure S2 Comparison of average CHC ratios per sex and species relative to the total peak area sums (percentages).

Table S1 CHC compounds identified from males and females of the four *Nasonia* species and *Trichomalopsis sarophagae* as well as their respective mean quantities.[†]

Table S2 Average manhattan distances based on the CHC divergence between males of the four *Nasonia* species (NG, NL, NO and NV) and the outgroup, *Trichomalopsis sarcophagae* (TS).

Table S3 Average manhattan distances based on the CHC divergence between females of the four *Nasonia* species (NG, NL, NO and NV) and the outgroup, *Trichomalopsis sarcophagae* (TS).

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