Entomologia Experimentalis et Applicata

FOUNDED 1958

DOI: 10.1111/eea.13106

A re-assessment of within-host mating behavior in the *Nasonia* species complex

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Accepted: 2 June 2021

Key words: hybrid, Hymenoptera, interspecific crosses, mating system, multiparasitization, *Nasonia*, parasitoid, prezygotic barrier, Pteromalidae, reproductive isolation, superparasitization, *Nasonia giraulti*

Abstract

Insects have a wide variety of mating systems. For parasitic wasps, mating sites are often confined to where their hosts occur and this mutual interaction therefore greatly affects the evolution of alternative mating strategies. Parasitoids of the genus *Nasonia* (Hymenoptera: Pteromalidae) – comprising the species *Nasonia vitripennis* (Walker), *Nasonia longicornis* Darling, *Nasonia giraulti* Darling, and *Nasonia oneida* Raychouhury & Desjardins – parasitize blowfly pupae and typically mate immediately after emergence of the females from the host puparium. However, in one *Nasonia* species, *N. giraulti*, mating predominantly occurs within the host. Here, we re-assess within-host mating (WHM) rates within the *Nasonia* genus, using laboratory lines and field-collected hosts, including *N. oneida* for which no data were available yet. We confirm that WHM rates are low in *N. vitripennis* and *N. longicornis*, but high in *N. giraulti*. WHM is rare in *N. oneida*, despite its sympatry and close phylogenetic relationship with *N. giraulti*. Multiparasitization experiments with *N. vitripennis* and *N. giraulti* resulted in lower WHM proportions of *N. giraulti*. To evaluate whether this was due to an exit hole in the host pupa created by *N. vitripennis* males – where an exit hole may result in emergence of *N. giraulti* females and possibly males prior to mating – we artificially created exit holes in the host parasitized by *N. giraulti* only. This also yielded a lower WHM proportion. Progeny analysis of interspecific crosses of *N. longicornis* and *N. oneida* with *N. giraulti*, generating hybrid female but non-hybrid male offspring, revealed that WHM is largely determined by the maternal species and, hence, by the species to which the male offspring belong. We conclude that WHM is predominantly a male-mediated trait in *N. giraulti*, resulting from males refraining from making exit holes and mating with females inside the host. We discuss how these findings can be used to evaluate whether WHM contributes to preventing species hybridization.

Introduction

The insect class is known for its broad variety of mating systems. Mating systems are influenced by the spatial and temporal distribution of females and males in nature because they determine the availability of potential mates (Thornhill & Alcock, 1983). As many insects are confined to their breeding areas, e.g., their host plants for oviposition, and have limited active dispersal capacity, they often have patchy distribution patterns. This is particularly true for parasitoids that lay their eggs in other insects. Due to their parasitic life style, parasitoid reproduction is strongly influenced by the distribution and life cycle of their insect hosts. This has a large effect on the chance that individuals encounter mating partners of their own species, as the distribution of hosts often determines where mating occurs (Godfray, 1994). Indeed, the strong dependence on the biology of their hosts has led to the evolution of parasitoid-specific mating systems (Godfray & Cook, 1997; Boulton et al., 2015). Many parasitoid species mate at their emergence site, where males compete for available females, which disperse subsequently. Often

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males and females differ in dispersal behavior because females need to search for new oviposition sites. In some species males have become sedentary and flightless. Furthermore, protandry – emergence of males before females – has evolved in many insects (Thornhill & Alcock, 1983), including parasitoid species. It gives the faster emerging males the opportunity to wait or actively search for the emerging females and mate with them upon emergence and before dispersal (van den Assem et al., 1980; Godfray, 1994). Another interesting behavior found in some parasitoids is mating before emergence from the host, called within-host mating (WHM). Albeit rare, this behavior has been observed in the egg parasitoids *Telonomus fariai* Haliday (da Costa Lima, 1928; Dreyfus & Breuer, 1944), *Trichogramma dendrolimi* Matsumura and *Trichogramma papilionis* Nagarkatti (Suzuki & Hiehata, 1985), and the blowfly pupal parasitoids *Nasonia giraulti* Darling and *Nasonia longicornis* Darling (Drapeau & Werren, 1999). It has been suggested that WHM can evolve in response to costly interspecific hybridization (Drapeau & Werren, 1999).

Parasitoid wasps of the genus *Nasonia* are well positioned to investigate the evolution of alternative mating strategies. The genus consists of a complex of four closely related species with overlapping distribution ranges. Three species occur in sympatry in eastern North America, namely *Nasonia vitripennis* (Walker), *N. giraulti*, and *Nasonia oneida* Raychouhury & Desjardins, although the distribution range of the recently discovered *N. oneida* is still poorly known (Raychoudhury et al., 2010). *Nasonia longicornis* occurs in sympatry with *N. vitripennis* in western North America. Despite their overlapping distribution ranges, hybridization between the species does not occur in nature. All species are infected with the endosymbiont *Wolbachia* that causes incompatibility between egg and sperm of different species (Breeuwer & Werren, 1990). In addition, species differ in courtship behavior resulting in various degrees of premating isolation (van den Assem & Werren, 1994; Bordenstein et al., 2000; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2018). Under laboratory conditions, the *Wolbachia* infection can be removed with antibiotics, which results in viable and fertile hybrid female offspring that can be used to study the genetics of species differences (Bordenstein & Werren, 2007; Loehlin et al., 2010; Diao et al., 2016).

Nasonia vitripennis is cosmopolitan and a generalist pupal parasitoid of various blowfly and flesh fly species that occur in bird nests and at carcasses, but the other three species are specialist parasitoids of *Protocalliphora* blowflies. They nevertheless often occupy similar host patches in nature and, when in sympatry, can parasitize the same individual hosts, a process defined as multiparasitism. For example, in a study at Ithaca (NY, USA), Grillenberger et al. (2009a) found that 29% (17 out of 58) of bird nests infested with *Nasonia* contained both *N. vitripennis* and *N. giraulti*, and 42% (35 of 84 hosts from three nests) of hosts contained offspring of both species. *Nasonia* species are gregarious and protandric, after eclosion males chew an exit hole in the host puparium and emerge from the host first, followed by the females. Males then can compete at the exit hole for access to the later emerging females. Population sex ratios are female-biased as a result of local mate competition (Werren, 1980, 1983; Grillenberger et al., 2009a,b; Steiner & Ruther, 2009; Moynihan & Shuker, 2011). Male *N. vitripennis* are flightless and remain at or near the host after emergence where they aggressively defend their position against other males until the females emerge. They often mate with the females emerging from their natal host, some of which may be their sisters depending on the number of females that oviposited on that host (Leonard & Boake, 2006; Mair & Ruther, 2018). *Nasonia giraulti* males do not show this behavior, but instead mate with the females within the host before chewing an exit hole to emerge (Drapeau & Werren, 1999). *Nasonia* females typically mate only once in nature (Burton-Chellew et al., 2007). Taken together, *N. vitripennis* and *N. giraulti* occur micro-sympatrically and mating interactions between them may occur frequently despite being costly in terms of post-mating isolation. Less is known about other interspecific interactions, such as between *N. vitripennis* and *N. longicornis* and the newly discovered species *N. oneida* (Raychoudhury et al., 2010).

Drapeau & Werren (1999) reported that WHM is frequent in *N. giraulti*, but absent in *N. vitripennis*, whereas *N. longicornis* has intermediate levels of WHM. *Nasonia oneida* (Raychoudhury et al., 2010) has not been tested for WHM behavior. Here, we revisit WHM as a possible mechanism to prevent interspecific mating, expanding on the earlier laboratory studies of Drapeau & Werren (1999) and Leonard & Boake (2006). We measure WHM proportions of the four known *Nasonia* species in various laboratory lines and in parasitized hosts collected directly from the field in areas of sympatry. Given that hosts can be parasitized by two species simultaneously (Grillenberger et al., 2009a), we further ask how WHM is affected in multiparasitized hosts containing both *N. vitripennis* and *N. giraulti* individuals. Finally, using laboratory strains that have been cured of their *Wolbachia* that allow for hybridization between species, we examine how the WHM trait is expressed in hybrids of two *Nasonia* species. We use our results to re-evaluate whether WHM is effective behavior to prevent interspecific mating between sympatric *Nasonia* species.

Materials and methods

Culturing and experimental conditions

Nasonia spp. mass cultures were maintained in plastic vials (9 cm high, 25 mm diameter) at 20 °C, L16:D8 photoperiod, and 45% r.h. in a climate-controlled chamber. During the experiments, wasps were cultured at 25 °C and L16:D8. Under these conditions, time until emergence is approximately 14 days for *N. vitripennis* and 16 days for *N. giraulti*, *N. longicornis*, and *N. oneida*, with males emerging about 1 day earlier than females. For all cultures and experiments, *Calliphora* spp. fly pupae were used as hosts for oviposition and development. Although *N. giraulti*, *N. longicornis*, and *N. oneida* prefer *Protocalliphora* as hosts in the field, they can readily be reared on *Calliphora* species. To obtain virgin wasps for crosses, males and females were collected separately in the pupal stage by opening host pupae about 2 days prior to expected wasp emergence.

Nasonia strains

Various strains comprising all four known *Nasonia* species that were available from laboratory cultures were used (Table 1). Four strains were cured of the cytoplasmic incompatibility-inducing *Wolbachia* bacteria (Breeuwer & Werren, 1990) by antibiotic treatment (tetracycline), indicated by the suffix 'TET' in the strain name. In addition, wasps of *N. vitripennis*, *N. giraulti*, and *N. longicornis* were collected from bluebird and tree swallow nests in the field in North America in 2011 (Table 1). No field lines could be obtained for *N. oneida* in 2011. From these collections, iso-female lines were established either from single females collected directly from the field, or from a single female that emerged from a field-collected host. These single female wasps were supplied with hosts and their offspring maintained in mass culture for about 2–3 generations prior to start of the experiment. Subsequently these lines were tested for WHM in the laboratory. For *N. giraulti*, 63 iso-female lines were established from 35 nests in Davidson (NC, USA), one iso-female line from one nest in Ithaca (NY), and 12 iso-female lines established from three nests in Waynesboro (VA, USA). For *N. vitripennis*, 11 iso-female lines were established from five nests in Davidson (NC), 30 iso-female lines from 24 nests in Ithaca (NY), 10 iso-female lines from one nest in Lancaster (PA, USA), and seven iso-female lines from two nests in Kingston (Ontario, Canada). For *N. longicornis*, four iso-female lines were established from one single nest in Ithaca (NY). *Nasonia longicornis* is endemic to western North America, but it has been released since the early 1980s as biocontrol agent and established in other parts of the continent (unpubl. data).

Measurement of within-host mating

To determine WHM proportions of different *Nasonia* species and strains, 1- or 2-day-old mated females were isolated from mass culture in a single plastic vial (6 cm high, 1 cm diameter) and provided with a single, fresh host that was replaced every 24 h for 5 days. Each parasitized host was incubated in a single plastic vial for 13 days; replication numbers are stated in the figures of the respective experiments. At the expected day of emergence, hosts were inspected at regular intervals (approximately every 15 min) during the lights-on phase. When the first

Table 1 Overview of *Nasonia* laboratory lines

Species	Strain	Collection site	Collection date	Additional information
N. vitripennis	HV-Mix3	Hoge Veluwe, The Netherlands	2001	A mixed outbred laboratory line established from five lines (van de Zande et al., 2014)
	SAL-8	Ithaca, NY, USA	2007	
	ITH-4C	Ithaca, NY, USA	2006	
	STDR-TET	Long-term laboratory strain		Red-eye mutant line, cured of *Wolbachia*
	RV-2	VA, USA	1986	
N. giraulti	NGPA-233F	PA, USA	1989	
	NGVA-1	VA, USA	1989	
	NGVA-2-TET	VA, USA	2006	Cured of *Wolbachia*
	NG-Mix	NY and VA, USA	2005 and 2006	A mixed outbred laboratory line established from five lines: RV2, NGVA-2, NGPA-233F, NGNY6A5, and NGVA-7B
	BR	Brewerton, NY, USA	1989	
N. oneida	NONY-11/36-TET	Brewerton, NY, USA	2005	Cured of *Wolbachia*
N. longicornis	RNLMN-8510-TET	MN, USA	1989	Cured of *Wolbachia*

emerged female was observed, the host was opened and 10 females were randomly collected from inside the host and placed individually in plastic vials. CO_2 anesthetization was used to prevent females from mating during collection and this was found not to influence the later behavior of the wasps (data not shown). Although this sampling approach ensures that no post-emergence mating occurs, it may lead to a slight underestimation of WHM proportions, as potentially ongoing WHM could be interrupted. The collected females were fed 10% sugar water for 24 h, and subsequently provided with two hosts for parasitization. Because *Nasonia* are haplodiploid, with females developing from fertilized diploid eggs and males from unfertilized haploid eggs, we could use offspring sex as a proxy for WHM. If daughters were found, the test female was scored as having mated inside the host prior to emergence. If only sons were produced, the test female was scored as virgin. Total number of offspring was disregarded, yet hosts without a single male were excluded. We scored the proportion of WHM by determining the number of test females (out of 10) that produced female offspring.

Parasitized hosts collected from the field were used to test whether WHM also occurs in hosts that have been parasitized in nature, or whether WHM is a behavior that only occurs in laboratory strains. For this, 95 parasitized *Protocalliphora* spp. hosts were collected from 39 bird nests at field sites in Ithaca (NY), in the summer of 2011. The WHM proportion of field-collected hosts was determined in a similar manner as described above for the laboratory strains, with the exception that not a subset of 10 but all females were collected from the hosts and setup for oviposition, as the number of females inside the field hosts varied and it was not always possible to collect 10 females.

Within-host mating after multiparasitization

To test whether *N. giraulti* mates within the host when another species is also present in that host, a multiparasitization experiment was conducted. A single host was simultaneously parasitized by a mated *N. giraulti* female (NGVA-1 strain) and a mated *N. vitripennis* female (STDR-TET strain). This yielded hosts containing *N. giraulti* females and males together with *N. vitripennis* females and males (n = 19). Offspring from *N. vitripennis* STDR-TET females were identified by their red eyes, instead of wild-type purple–brown eyes of the *N. giraulti* NGVA-1 strain. Although these two species differ in developmental time in single-species cultures, in these multiparasitized hosts female wasps of both species emerged simultaneously. After the first female, irrespectively of its species identity, emerged from a test host, up to 10 *N. giraulti* females were collected from the host to determine their WHM proportions.

To test whether WHM behavior of *N. giraulti* females is influenced by the presence of an exit hole in the host – in the experiment above created by prior emerging *N. vitripennis* males – an artificial exit hole was created in the fly puparium parasitized by *N. giraulti* with a needle 3 days prior to emergence of the wasps. This was performed separately for the NGVA-1 (n = 24), NGPA-233F (n = 16), and BR (n = 9) *N. giraulti* strains. Within-host mating proportions were scored per strain as described above, by opening a host after the first female emerged from it and up to 10 females were collected.

Sex-specific expression of within-host mating behavior

To investigate whether WHM is a male- or female-mediated trait, interspecific crosses were conducted, which allowed for combining a pure-species male genotype with a two-species mixed female genotype. For this, laboratory lines were used that were cured of their *Wolbachia* bacteria: the *N. giraulti* NGVA-2-TET line, the *N. longicornis* RLMN-8510-TET line, and the *N. oneida* NONY-11/36-TET line (Table 1). A virgin female of one species was mated to a male of another species to generate hybrid females (for cross types and sample sizes, see Figure 4's caption). Intraspecific crosses were set up as control. Two days after mating, females were provided with hosts. Owing to haplodiploidy, parasitized hosts contained F1 hybrid females (with a 50:50 genomic composition of the two species) that developed from fertilized eggs, and haploid non-hybrid males of the maternal species that developed from unfertilized eggs.

Statistical analysis

Data were analyzed by fitting generalized linear mixed models with binomial error distributions. The WHM proportions of factor levels were compared to the model grand mean using 'sum to zero' contrasts, and female ID as random factor. In the first experiment, species strain and sampling location were analyzed as nested within species with random intercept. Stated model estimate values indicate the effect size of factor levels on WHM proportions; where factor levels with positive estimates have an above average score and factor levels with negative estimates a below average score. Differences between factor levels were analyzed in multiple comparisons with Tukey contrasts and Holm adjustment. Data of intra- and interspecific crosses were analyzed regarding the genetic background of F1 males and the hybridization status of F1 females with user-defined contrasts and single-step adjustment. All statistical analyses were performed in R v.3.6.3 (R

Development Core Team, 2020), using the packages *lme4* v.1.1-21 (Bates et al., 2015) and *multcomp* v.1.4-12.

Results

Within-host mating in laboratory and field lines

Within-host mating proportions were scored in four *Nasonia* species including eight laboratory strains (Figure 1A). All four species significantly differed in their WHM proportion from the model grand mean, where *N. giraulti* had an above average and *N. vitripennis*, *N. longicornis*, and *N. oneida* a below average WHM proportion (GLMM estimates *N. giraulti*: 3.82, *N. vitripennis*: −7.08, *N. longicornis*: −2.60, and *N. oneida*: −1.54). *Nasonia oneida* exhibited the highest variation in WHM proportions. Pairwise comparison revealed that differences between all four species were significant with exception of *N. oneida* vs. *N. longicornis* (Figure 1A, Table 2). Two of the three tested *N. vitripennis* strains (SAL-8 and ITH-4C) showed complete absence of WHM. Overall, strain identity (within species) had no impact on WHM, except that *N. giraulti* strain NG-Mix had a significantly lower WHM proportion, but this was largely caused by a single replicate.

WHM proportions of field lines of *N. vitripennis*, *N. giraulti*, and *N. longicornis* were consistent with the laboratory lines tested (Figure 1B); *N. giraulti* had the highest WHM proportion compared to the model grand mean (GLMM estimate = 3.87, Z = 14.25, P<0.001) and *N. vitripennis* had the lowest WHM proportion (GLMM estimate = −4.15, Z = −14.29, P<0.001). *Nasonia longicornis* individuals showed the highest variation in WHM proportion which did not differ from the model grand mean (GLMM estimate = −0.53, Z = −1.08, P = 0.28). All three species differed in WHM proportion from each other in pairwise comparisons (Figure 1B, Table 2).

Within-host mating in field-collected hosts

WHM proportions of field-collected hosts parasitized by either *N. giraulti* or *N. vitripennis* were consistent with the WHM proportions of the field lines (Figure 2). Again, *N. giraulti* had the highest WHM proportion (model grand mean: GLMM estimate = 1.64, Z = 1.87, P = 0.061; note that only five collected females contribute to the data). In *N. vitripennis*, WHM was nearly absent (GLMM estimate = −3.63, Z = −7.78, P<0.001). *Nasonia giraulti* and *N. vitripennis* differed in their WHM proportions (Tukey contrast with Holm adjustment: estimate = 5.260 ± 1.054, Z = 4.99, P<0.001; Figure 2).

Within-host mating after multiparasitization

To test whether *N. giraulti* females also mate inside the host when that host has been multiparasitized, WHM

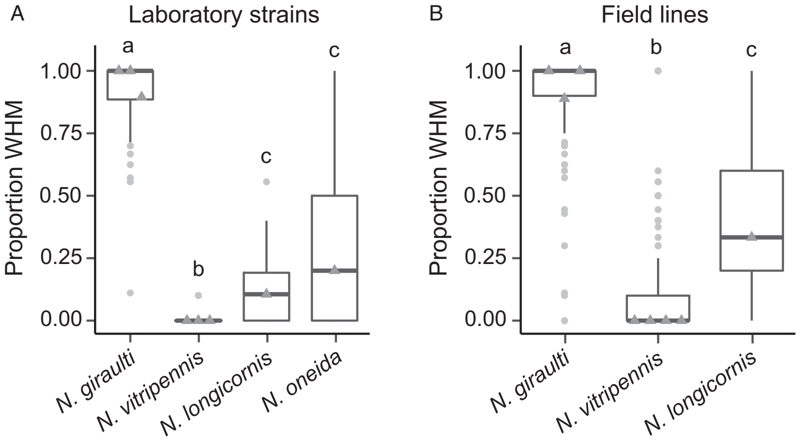

Figure 1 Within-host mating (proportion WHM) in (A) laboratory strains and (B) recently field-collected lines of various *Nasonia* species. In (A) the triangles indicate medians of various strains within species, for *N. giraulti* (from left to right): NGPA-233F (n = 10), NGVA-1 (n = 15), and NG-Mix (n = 23); for *N. vitripennis*: HV-MIX3 (n = 32), ITH-4C (n = 15), and SAL-8 (n = 24); for *N. longicornis*: RNLMN-8510-TET (n = 22); and for *N. oneida*: NONY-11/36-TET (n = 37). In (B) the triangles are medians of various sampling locations within species, for *N. giraulti* (from left to right): Davidson (n = 146), Ithaca (n = 3), and Waynesboro (n = 33); for *N. vitripennis*: Davidson (n = 46), Ithaca (n = 99), Lancaster (n = 23), and Kingston (n = 24); and for *N. longicornis*: Ithaca (n = 23). The boxplots represent the 25th and 75th percentiles, the whiskers 1.5 × the interquartile range, and the dots are outliers. The thicker lines indicate the median of the data groups. Means within a panel capped with different letters are significantly different (Tukey: P<0.05).

Table 2 Within-host mating in laboratory and recently field-collected *Nasonia* species. Results from simultaneous tests for general linear hypotheses with step-wise adjustment

Comparison		Estimate	Z	P
Laboratory lines	*N. vitripennis* vs. *N. giraulti*	−10.898 ± 1.326	−8.218	<0.001
	N. longicornis vs. *N. giraulti*	−6.419 ± 1.126	−5.700	<0.001
	N. oneida vs. *N. giraulti*	−5.354 ± 1.073	−4.989	<0.001
	N. longicornis vs. *N. vitripennis*	4.479 ± 1.279	3.503	<0.001
	N. oneida vs. *N. vitripennis*	5.543 ± 1.239	4.473	<0.001
	N. oneida vs. *N. longicornis*	1.064 ± 1.160	0.918	0.36
Field lines	*N. vitripennis* vs. *N. giraulti*	−8.020 ± 0.474	−16.924	<0.001
	N. longicornis vs. *N. giraulti*	−4.399 ± 0.565	−7.786	<0.001
	N. vitripennis vs. *N. longicornis*	−3.621 ± 0.566	−6.403	<0.001

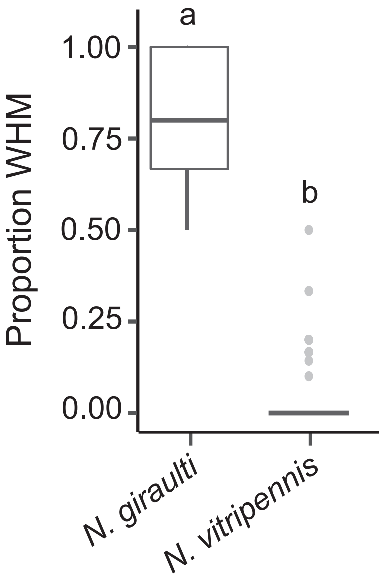

Figure 2 Within-host mating (proportion WHM) in field-collected host pupae of two *Nasonia* species. Among a total of 95 host pupae five were parasitized by *N. giraulti* and 90 were parasitized by *N. vitripennis*. The boxplots represent the 25th and 75th percentiles, the whiskers 1.5× the interquartile range, and the dots are outliers. The thicker lines indicate the median of the data groups. Means capped with different letters are significantly different (Tukey's test: P<0.05).

proportion was measured in hosts containing males and females of both *N. giraulti* and *N. vitripennis*. Females from hosts parasitized solely by *N. giraulti* had a higher WHM proportion (GLMM estimate = 9.05, Z = 4.02, P<0.001) and females collected from multiparasitized hosts had a lower WHM proportion (GLMM estimate = −7.54, Z = −4.02, P<0.001). Notably, the *N. giraulti* females and males emerged from the host simultaneously with the *N. vitripennis* females, which is almost 2 days earlier than expected for *N. giraulti*. These results clearly indicate that *N. giraulti* individuals left the puparium through the exit hole created by the faster developing *N. vitripennis* males.

To further examine this, an additional treatment was included, where host pupae parasitized by *N. giraulti* were provided with artificially created exit holes. In three *N. giraulti* lines tested, WHM proportions of females from such manipulated hosts were even lower compared to the model grand mean (GLMM estimate = −9.14, Z = −5.38, P<0.001). Pairwise comparisons revealed significant differences in WHM between single species and multiparasitization, between single species and artificial exit hole, but not between multiparasitization and artificial exit hole (Figure 3, Table 3). These results indicate that WHM proportions depend on the absence of an exit hole in the host, and suggest that *N. giraulti* males are able to mate with females inside the host by refraining from making exit holes.

Sex-specific effect on within-host mating behavior

Reciprocal crosses between species were conducted to investigate which sex determines WHM behavior. WHM proportions were measured for F1 hybrid and purebred females from three species pairs. In crosses between *N. giraulti* and *N. longicornis* (L × G and G × L crosses), F1 females from hosts containing *N. giraulti* males (from the L × G cross) had a significantly higher WHM proportion than F1 females from hosts where *N. longicornis* males (from the G × L cross) were present (Figure 4A, Table 4). If the F1 male species was kept the same, pure and hybrid F1 females had similar WHM proportions for *N. giraulti* males. In contrast, when *N. longicornis* males were present hybrid females had a significantly higher WHM proportion than pure females (Figure 4A, Table 4).

Also in the *N. giraulti* × *N. oneida* crosses (G × O and O × G), F1 females had a higher WHM proportion if *N. giraulti* males were present (O × G cross) in the host

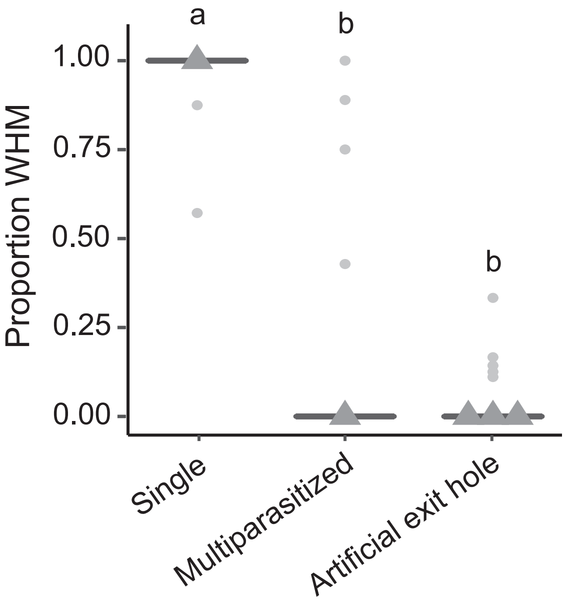

Figure 3 Within-host mating (proportion WHM) of *Nasonia giraulti* after multiparasitization and artificial exit hole, in hosts solely parasitized by *N. giraulti*, co-parasitized by *N. giraulti* and *N. vitripennis* (strain STDR-TET), or parasitized by *N. giraulti* where 3 days prior to expected emergence an artificial exit hole was created in the host pupae. Triangles indicate medians of *N. giraulti* strains (from left to right), single: NGVA-1 (n = 15); co-parasitized: NGVA-1 (n = 19); and artificial exit hole: NGVA-1 (n = 24), NGPA (n = 16), and BR (n = 9). The lines represent the median of the data groups and the dots are outliers exceeding 1.5× the interquartile range (25th and 75th percentiles are equal to median). Means capped with different letters are significantly different (Tukey's test: P<0.05).

Table 3 Within-host mating phenotype of *Nasonia giraulti* in multiparasitized hosts and hosts inflicted with artificial exit hole. Results from simultaneous tests for general linear hypotheses with Tukey contrasts with Holm adjustment

Comparison	Estimate	Z	P
Single vs. multiparasitized	16.593 ± 3.438	4.827	<0.001
Single vs. exit hole	18.194 ± 3.290	5.529	<0.001
Exit hole vs. multiparasitized	−1.601 ± 1.809	−0.885	0.38

pupa compared to if *N. oneida* males were present (G × O cross) (Figure 3B, Table 3). Females being hybrid also had a significant effect on WHM, in the presence of *N. giraulti* males the G/O and O/G females had a lower WHM proportion and in the presence of *N. oneida* males they had a higher WHM proportion compared to the respective purebreds (Figure 4B, Table 4).

Overall, the *N. longicornis* × *N. oneida* crosses (L × O and O × L) showed the lowest WHM proportion, as compared to the other species crosses, where neither the F1 male species present in the host pupa nor the pure vs. hybrid status of the F1 female had a significant effect on the WHM proportion (Figure 4C, Table 4). Overall, these results show that WHM proportions are strongly determined by male species, but are also affected by the specific species combination of female genotype.

Discussion

Our data from *N. vitripennis*, *N. longicornis*, and *N. giraulti* generally confirm previous WHM observations by Drapeau & Werren (1999) and Leonard & Boake (2006). However, *N. giraulti* in our study tends to have higher WHM proportions than reported by Drapeau & Werren (1999). This is most probably the result of different scoring methods. Our method used anesthetization of females, which ensures that all females are tested at one specific moment, whereas Drapeau & Werren's method could include younger females that were not yet receptive to mating. Newly collected *N. vitripennis* and *N. giraulti* wasp lines, as well as hosts collected directly from the field, yielded WHM proportions similar to those in established laboratory lines. This makes it unlikely that WHM is a behavior affected by fly host species or adapted to the laboratory rearing situation, but instead also occurs frequently in nature for *N. giraulti*, but not for *N. vitripennis*.

Within-host mating proportions were found to be high in *N. giraulti*, but low in *N. longicornis* and *N. oneida* laboratory lines. This study is the first to report WHM rates in *N. oneida* and although only one laboratory strain was examined, we have as of yet no indications that maintenance of *Nasonia* lines in the laboratory alters WHM behavior. Interestingly, *N. giraulti* and *N. oneida* occur sympatrically, with *N. vitripennis* in eastern North America, and they differ widely in WHM proportions despite their recent divergence (Raychoudhury et al., 2010). This suggests that the WHM trait can rapidly be gained or lost. *N. oneida* females discriminate strong against *N. vitripennis* males, whereas *N. giraulti* females do not (Giesbers et al., 2013). This could indicate the evolution of an alternative strategy to prevent hybridization with *N. vitripennis*. Raychoudhury et al. (2010) found *N. oneida* females to discriminate strongly against *N. giraulti* males as well, but this was not confirmed by Giesbers et al. (2013). Although these results may indicate differences between strains, more *N. oneida* strains would need to be tested. A lower degree of interspecific mate discrimination of *N. oneida* toward *N. giraulti* than toward *N. vitripennis* may result from fewer contacts due to high WHM in *N. giraulti*.

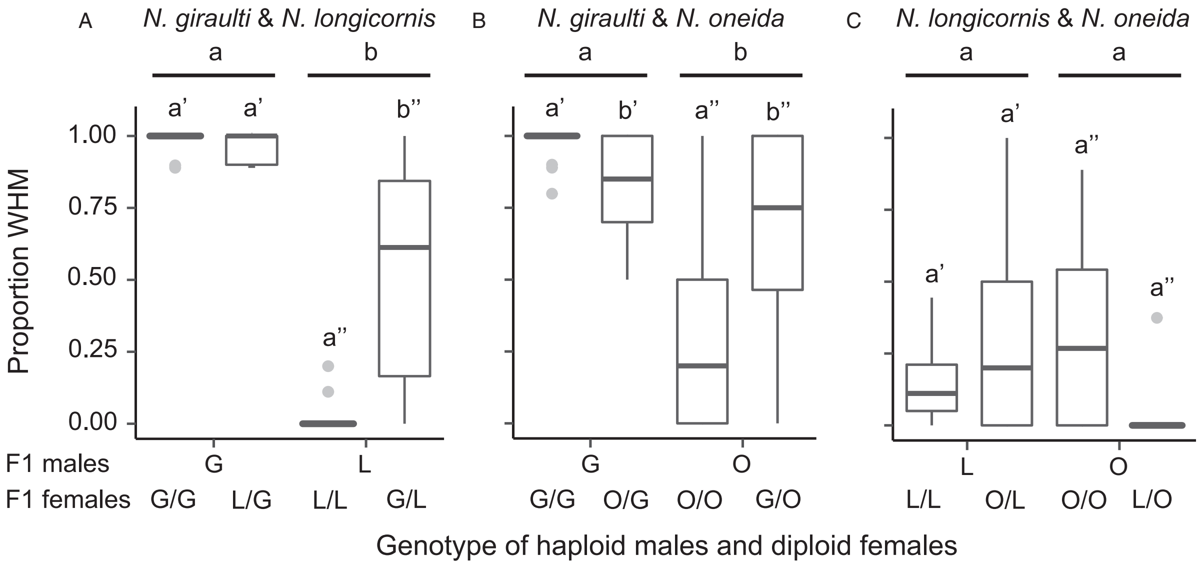

Figure 4 Within-host mating (proportion WHM) in intra- and interspecific crosses of *Nasonia* species: (A) *N. giraulti – N. longicornis*, G × G (n = 10), L × G (n = 14), L × L (n = 12), G × L (n = 14). (B) *N. giraulti – N. oneida*, G × G (n = 24), O × G (n = 14), O × O (n = 37), G × O (n = 31). (C) *N. longicornis – N. oneida*, L × L (n = 15), O × L (n = 18), O × O (n = 37), L × O (n = 8). In F1 females, the first character indicates the origin of the paternal genome, whereas the second character indicates the origin of the maternal genome. The boxplots represent the 25th and 75th percentiles, the whiskers 1.5× the interquartile range, and the dots are outliers. The thicker lines indicate the median of the data groups. Means within a panel capped with the different letters are significantly different (Tukey's test: P<0.05). The primes indicate groups of pairwise comparisons.

Table 4 Within-host mating in intra- and interspecific crosses of *Nasonia* species. Results from simultaneous tests for general linear hypotheses with step-wise adjustment

Comparison		Estimate	Z	P
N. giraulti & *N. longicornis*	*N. giraulti* male vs. *N. longicornis* male	−13.136 ± 1.799	−7.302	<0.001
	N. giraulti male: purebred vs. hybrid	−0.923 ± 1.146	−0.806	0.78
	N. longicornis male: purebred vs. hybrid	4.961 ± 1.085	4.553	<0.001
N. giraulti & *N. oneida*	*N. giraulti* male vs. *N. oneida* male	−8.124 ± 1.207	−6.731	<0.001
	N. giraulti male: purebred vs. hybrid	−3.223 ± 0.986	−3.270	0.003
	N. oneida male: purebred vs. hybrid	2.980 ± 0.604	4.938	<0.001
N. longicornis & *N. oneida*	*N. longicornis* male vs. *N. oneida* male	−1.923 ± 1.194	−1.610	0.26
	N. longicornis male: purebred vs. hybrid	1.215 ± 0.644	1.887	0.15
	N. oneida male: purebred vs. hybrid	−2.242 ± 1.002	−2.239	0.067

However, these explanations remain speculative without more information on the degree of overlap in spatial and temporal distribution of these species in nature.

A large effect of the male species present in the host was found in multiparasitized hosts: significantly fewer *N. giraulti* females mated within the host, when both *N. vitripennis* and *N. giraulti* males were present. A likely explanation for this result is that *N. vitripennis* males chew an exit hole in the puparium wall soon after eclosion, through which the *N. giraulti* males and females also emerge. This would shorten the time period that the *N. giraulti* males can mate with *N. giraulti* females inside the host. To test the validity of this hypothesis, artificial exit holes were created in hosts that were parasitized by *N. giraulti* only. This also resulted in a significant decrease of WHM, indicating that the timing of the creation of exit holes by males is an important factor in determining WHM behavior. Apparently, the high WHM rate in *N.*

giraulti results from males that mate females inside the host by waiting to create an exit hole. Indeed, Mair & Ruther (2018) observed that it is often the females that chew the exit hole and emerge first in this species.

Interspecific crosses between species that differ in WHM were conducted to determine which sex induces this behavior. In order to reduce the effects of interspecific mate discrimination, only species combinations were used that show little pre-zygotic isolation (Giesbers et al., 2013). This excluded testing the combination of *N. giraulti* and *N. vitripennis* that differ strongest in WHM proportions, because *N. vitripennis* females, as well as *N. vitripennis* × *N. giraulti* hybrid females, discriminate strongly against *N. giraulti* males; successful mating is therefore hard to obtain. Crosses between *N. giraulti* and *N. longicornis*, as well as between *N. giraulti* and *N. oneida*, yielded F1 hybrid females with intermediate WHM proportions. Although this may suggest additive genetic variation for WHM, caution needs to be exerted because of the complex nature of the species interactions in haplodiploid gregarious parasitoids such as *Nasonia*. Direct comparison of the two reciprocal hybrid crosses is hampered by changes in three variables at the same time, i.e., the male species present in the host, hybrid female choice toward that male species, and harmful effects on fitness of the hybrid females, as well as the added possibility of interactions between these three variables. Crosses between *N. giraulti* and *N. longicornis* in which the male was *N. giraulti* resulted in higher WHM proportions irrespective of female genotype, which indicates an effect of the male species present in the host, consistent with the observation that *N. giraulti* performs more WHM than *N. longicornis*. In the hybrid cross between *N. oneida* and *N. giraulti* similar significantly elevated WHM rates were found when *N. giraulti* males were present in the host compared to *N. oneida* males. There were, however, also differences between female genotypes of particular species composition. Together, these results indicate that WHM is a trait mediated by both males and females.

There are, however, alternative explanations. It could be that mate discrimination of hybrid females has affected the results. Although we have no data on mate discrimination in *N. giraulti* × *N. longicornis* hybrids against either *N. longicornis* or *N. giraulti* males to verify this, mate discrimination between pure *N. giraulti* and *N. longicornis* is low (Giesbers et al., 2013; Buellesbach et al., 2014). In addition, *N. oneida* females discriminate strongly against *N. giraulti* males, but the strong discrimination trait is recessive (Raychoudhury et al., 2010). Another possible complication is reciprocal cross effects, even though the hybrid female offspring of two reciprocal crosses are identical in terms of nuclear genome composition they may differ due to maternal or cytoplasmic effects. Mitochondrial and/or epigenetic effects on hybrid male mating behavior and female mate discrimination have been reported in *Nasonia* (Beukeboom & van den Assem, 2001, 2002). These effects are likely not strong enough to explain the large differences in WHM proportions in our hybrid crosses, as mate discrimination rates between the pure species are low and rather similar for both reciprocal crosses (Giesbers et al., 2013).

The evolution of WHM should be considered in the context of other relevant aspects of the mating system. First, it is facilitated by protandry – the faster development of males – than females. Although both species are protandric, the *N. giraulti* mating system is different from that of *N. vitripennis*, as *N. giraulti* males do not emerge upon eclosion but remain in the host to mate and exit later. Moreover, WHM appears to be linked with site fidelity, because in contrast to *N. giraulti*, *N. vitripennis* does not exhibit WHM and males aggressively defend their emergence site (Leonard & Boake, 2006; Mair & Ruther, 2018). Also *N. longicornis* shows high site fidelity and aggression at similar levels as *N. vitripennis* (Leonard & Boake, 2006) and low WHM proportion. Interestingly, *N. vitripennis* males are flightless but appear to use their short wings in aggressive encounters, whereas *N. giraulti* males have long wings and can fly, and seem to disperse more often from their natal patch (Leonard & Boake, 2006; Mair & Ruther, 2018). Post-mating dispersal by males may allow them to find additional mates, but this depends on whether any females disperse unmated or remate, information that is not available from natural populations.

How the differences in mating strategies between *N. giraulti*, *N. oneida*, and *N. vitripennis* work out when these species occupy the same host simultaneously is not clear. Our multiparasitization experiment of *N. giraulti* and *N. vitripennis* revealed that hosts that were simultaneously parasitized by both species resulted in a lower WHM proportion for *N. giraulti* females than hosts that were exclusively parasitized by *N. giraulti*. Hence, a higher proportion of unmated *N. giraulti* females may leave the host and run the risk of being mated by *N. vitripennis* males upon emergence. Drapeau & Werren (1999) proposed that WHM evolved in *N. giraulti* as a means of preventing hybridization with *N. vitripennis* in areas of sympatry. Grillenberger et al. (2009) showed that multiparasitization may occur frequently in nature. Our results indicate that WHM of *N. giraulti* is reduced when both species develop in the same host and, thus, prevents interspecific mating only to a limited extent. The strength of selection pressure for preventing hybridization may critically depend on the frequency at which multiparasitization occurs both at the level of individual hosts and host patches. Solving this question would require detailed studies of spatial and temporal overlap of species and quantifying interspecific interactions. The evolution of inter-sexual and inter-species mate choice remains a topic of considerable interest (Shuker & Simmons, 2014). The *Nasonia* species complex appears well suited for such study.

Acknowledgments

In loving memory of our colleague Silvia Paolucci; we are grateful for her support in the field collection and laboratory work, and for the many fruitful discussions during the implementation of this study.

Author Contribution

Monika Trienens: Data curation (equal); Formal analysis (lead); Writing-review & editing (lead). Maartje C.W.G. Giesbers: Data curation (equal); Formal analysis (equal); Investigation (lead); Writing-original draft (lead). Bart A. Pannebakker: Formal analysis (supporting); Supervision (equal); Writing-original draft (equal); Writing-review & editing (equal). Louis van de Zande: Supervision (equal); Writing-original draft (equal); Writing-review & editing (equal). Leo W. Beukeboom: Project administration (lead); Supervision (equal); Writing-original draft (lead); Writing-review & editing (lead).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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