CHAPTER 2

Prezygotic Isolation in the Parasitoid Wasp Genus Nasonia

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ABSTRACT

_Nasonia_ (Hymenoptera: Pteromalidae) are small haplodiploid parasitoids of flesh- and blowfly pupae that have become model organisms for speciation research. The genus consists of four closely related species that harbor species-specific *Wolbachia* bacteria that cause postzygotic reproductive isolation. Antibiotic curing allows for interspecific crosses and genetic exchange between species which, together with haploidy of males, facilitates genetic analysis of fitness traits. In this chapter we synthesize the current knowledge on the different prezygotic isolation factors that act in the _Nasonia_ genus, and on the genetic basis of these traits. A major prezygotic isolation factor is courtship behaviour. Species differ in male courtship behaviour, and there is large variation in interspecific MD depending on species pair. We summarize data on the strength of prezygotic isolation barriers between all possible species pairs and present new data on MD in choice and no-choice experiments. In tests of reinforcement, we found no stronger female MD of _N. vitripennis_ strains occurring in microsympatry with _N. giraulti_ compared to that of allopatric _N. vitripennis_ strains. Additionally, we present data on the significance of cuticular hydrocarbon profiles for assortative mating in males and discuss other factors that may be involved in prezygotic isolation, including pheromone communication, WHM and sneaking behaviour.
INTRODUCTION

The Biological Species concept states that species are ‘groups of interbreeding natural populations that are reproductively isolated from other such groups’ (Mayr 1942). When a reproductive barrier between two populations arises, local adaptation and drift can lead to divergence of these populations, and eventually to speciation (Mayr 1963). These reproductive isolation barriers can be divided into those that act before fertilization, i.e. prezygotic isolation, and those that act after fertilization, i.e. postzygotic isolation (Dobzhansky 1935, Mallet 1998). Prezygotic isolation barriers typically manifest prior to mating, for example through temporal or geographic isolation between individuals, morphological differences between mating partners, or behavioural isolation (Kondrashov and Shpak 1998, Coyne and Orr 1998). However, post-mating prezygotic isolation can also occur, for example through incompatibilities between egg and sperm (e.g. Shaw et al. 1994).

Assortative mating, i.e. preferred mating among individuals of the same species over matings between individuals of different species, is arguably the most prevalent, or at least best studied, form of prezygotic isolation. Despite the fact that prezygotic reproductive barriers do not always lead to completely isolated species for prolonged periods of time, behavioural isolation can apparently evolve rapidly (Coyne and Orr 1997, 2004). The evolution of behavioural barriers can be studied especially well in young species complexes, where hybridization can still occur and a full barrier to gene flow has not yet been established (e.g. Coyne et al. 2002, Gow et al. 2006). A well-known, and frequently studied, behavioural barrier is female interspecific MD, in which females of a particular species show strong assortative mating. This behaviour can be strengthened by reinforcement, a process in which prezygotic isolation barriers are strengthened by natural selection against unfit hybrids (Dobzhansky 1940, Servedio and Noor 2003).

Despite the attention for behavioural reproductive isolation in the process of speciation, the genetic basis of traits that play a role in maintaining and/or creating prezygotic isolation is not well known (e.g. Arbuthnott 2009). We do not have good answers yet to many questions, such as whether behavioural isolation is based on few genes with large effects or many genes with small effects, whether most behavioural changes cause reproductive isolation (hence behavioural genes being speciation genes, sensu Orr et al. 2004) or come into being after the speciation event (hence contributing to species differences). Such knowledge is required to understand how selection and drift may cause changes in reproductive isolation between populations in the course of evolution.

In this chapter we present the results of many years of research on speciation in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae). *Nasonia* have been used extensively for research on speciation and adaptation for a couple of decades now (e.g. Werren 1983, Pultz and Leaf 2003, Leonard and Boake 2006), and their haplodiploid sex
determination system makes them very well suited for genetic analyses (Beukeboom and Desplan 2003, Werren and Loehlin 2009, Werren et al. 2010). The complete genome sequences are available for all four species, making this young species complex ideal for genomic studies on complex traits (Werren et al. 2010). In this chapter, we present an overview of the knowledge on prezygotic isolation between the *Nasonia* species. We summarize previously published data, and provide new information that has been gathered by our research groups in the course of time.

**NASONIA WASPS**

*Nasonia* (Hymenoptera: Pteromalidae) is a genus of 2-3 mm sized parasitoid wasps (Figure 2.1) that lay their eggs in pupae of various fly species, such as Protocalliphora, that occur in bird nests and on carcasses (Whiting 1967, Darling and Werren 1990). A clutch typically consists of 20-30 eggs depending on host size (Charnoy and Skinner 1984). Like all Hymenoptera, *Nasonia* has a haplodiploid mode of reproduction (Whiting 1967). Males are uniparentally produced and haploid; they develop from unfertilized eggs. Females have a mother and a father, are diploid and develop from fertilized eggs. Females store sperm after mating and can control the process of egg fertilization (Holmes 1972, Werren 1984, King and Skinner 1991). In the field, clutches typically contain mostly fertilized (female)
eggs (Whiting 1967, Werren 1983), of which the developmental time is approximately 14-16 days at 25°C. Male offspring emerges first and stay at the natal patch where they mate with emerging females, that can both be their sisters and offspring of other founding parental females, if present (Whiting 1967, van den Assem et al. 1980, Leonard and Boake 2006). Females typically mate only once and disperse in search of new host patches, but they may become receptive a second time 24 hours after mating (van den Assem and Visser 1976, Grillenberger et al. 2009a). The combination of these life history traits makes Nasonia a prime example of local mate competition (Hamilton 1967, Werren 1980, 1983).

The Nasonia genus consists of four species: N. vitripennis, N. giraulti, N. longicornis (Darling and Werren 1990), and the recently discovered species N. oneida (Raychoudhury et al. 2010a). N. vitripennis diverged from its sister species approximately 1.0 million years ago (Campbell et al. 1993), while N. longicornis and N. giraulti diverged approximately 0.5 million years ago (Raychoudhury et al. 2010b), and N. oneida diverged from N. giraulti 0.4 million years ago (Raychoudhury et al. 2010a). N. vitripennis is a cosmopolitan species and occurs in sympathy with N. giraulti and N. oneida in eastern and with N. longicornis in western North America, respectively (Darling and Werren 1990; Raychoudhury et al. 2010a, Figure 2.2). N. longicornis is spatially isolated from N. giraulti and N. oneida, but N. vitripennis is the only species that occurs in true allopatry outside North America. In areas of sympathy, the opportunity for hybrid matings is potentially high because the species are morphologically very similar (Darling and Werren 1990), they can occupy the same host patch or even hatch from the same host pupa (Darling and Werren 1990, Grillenberger et al. 2009b) and they can hybridize in the laboratory. Exact frequencies of hybrid matings under natural conditions have not been investigated so far. However, there is strong postzygotic reproductive isolation between most of the species in nature due to infection with species-specific strains of Wolbachia bacteria that cause cytoplasmic incompatibility and hybrid breakdown (Breeuwer and Werren 1990, 1995). Such incompatibility, between egg and sperm that are infected with different Wolbachia strains, results in improper condensation, eventual damage and subsequent loss of the paternal chromosomes in fertilized eggs (Ryan and Saul 1968, Breeuwer and Werren 1990, Tram and Sullivan 2002). The resulting haploid embryos may either die or develop into haploid males (Breeuwer and Werren 1990, Bordenstein and Werren 1998, Bordenstein et al. 2003, Tram et al. 2006). Interspecific matings therefore usually result in all-male progenies, the haploid males being non-hybrid because of haplodiploidy, and sometimes in reduced offspring numbers. The strength of cytoplasmic incompatibility varies between species pairs, and is generally proportional to their divergence times. The Wolbachia strains of N. vitripennis induce complete incompatibilities with those of all three other species, N. giraulti and N. longicornis strains are partially incompatible (Breeuwer and Werren 1990, Bordenstein et al. 2001), but the Wolbachia strains from N. oneida are fully compatible with those of N. giraulti and partially
compatible with those of *N. longicornis* (see Raychoudhury et al. 2010a and Figure 2.4 for details). Antibiotic curing from the *Wolbachia* bacteria allows for heterospecific crosses in the laboratory that yield viable and (partially) fertile F1 females and F2 males, despite other pre- and postzygotic barriers that are present between the different species pairs at varying degrees.

Apart from cytoplasmic incompatibilities reducing the number of hybrid offspring, F2 hybrid males, between *N. vitripennis* and both *N. giraulti* and *N. longicornis*, suffer from strong postzygotic hybrid breakdown (Breeuwer and Werren 1995, Gadau et al. 1999, Niehuis et al. 2008, Clark et al. 2010, Koevoets et al. 2012) Together, through these postzygotic isolation mechanisms a substantial fitness disadvantage can be expected from females who permit courtship from and copulations with heterospecific males. Similarly, from the male perspective, investment in interspecific copulations means a complete absence of mating success in terms of paternal genetic contribution to the offspring (Bordenstein and Werren 2007). Thus, selection pressure towards MD and assortative mating is a likely consequence, favoring the ability to discriminate conspecific from heterospecific mating partners (Butlin 1987). In this chapter we present the current knowledge on prezygotic isolation mechanisms in the *Nasonia* genus and consider whether the observed species differences could have evolved in response to maladaptive hybridization.

![Geographical distribution of the four Nasonia species.](image)

**Figure 2.2** Geographical distribution of the four *Nasonia* species. *N. oneida* was recently discovered, and has as yet only been found in the region of Ithaca (NY). *N. vitripennis* is a cosmopolitan species.
COURTSHIP AND MATING BEHAVIOUR

The *Nasonia* species differ in male courtship behaviour and can be discerned by their species-specific alterations of a common courtship pattern (van den Assem and Werren 1994, Drapeau and Werren 1999). This difference alone is not sufficient to prevent interspecific matings. In fact, it is still not well known to what extent these differences in male courtship behaviour are perceived by females to affect their receptivity. Moreover, it is unclear whether male courtship behaviour has mostly been shaped by intraspecific sexual selection as suggested by Barrass (1976) and Jachmann and van den Assem (1996), or has also been modulated by interspecific interactions. In *Nasonia*, female reproductive behaviour appears to induce a particularly stronger isolation than male behaviour does. In addition to information obtained from male courtship behaviour, females might discriminate against males of other species using species-specific differences in sex pheromones, aphrodisiacs and sound (Ruther et al. 2009, Robertson et al. 2010, Niehuis et al. 2011).

*Nasonia* has been extensively used for behavioural studies for several decades (Barrass 1960, van den Assem and Visser 1976; van den Assem 1986, Jachmann and van den Assem 1993; van den Assem and Werren 1994), including some studies of the underlying genetics of behaviour (Beukeboom and van den Assem 2001, 2002, Veltzhuisk et al. 2005). In this chapter, female interspecific mate discriminating (MD) behaviour is considered to be the main prezygotic isolation mechanism that separates the *Nasonia* species. This behaviour, in which females of a species are reluctant to mate with heterospecific males, can be influenced by a number of different cues that are embedded in the mating sequence. However, research of the previous years focused almost solely on male courtship behaviour and female response. In laboratory observations, the start of a mating sequence is marked by the latency time. This is the time it takes the male and female to approach each other, and defined as the time period between the moment of introducing the two partners to the mating arena and the moment that the male mounts the female and takes up the courtship position on top of the female. Following some evidence from van den Assem et al. (1980), Ruther et al. (2007) showed that *Nasonia vitripennis* males release a sex pheromone from their abdomen to attract females. This pheromone can be considered as a cue for the female, and might determine the duration of the latency time.
Figure 2.3: Schematic representation of the courtship pattern of Nasonia. Pictures by Michael Clark.
After the male has mounted the female, the male courtship behaviour of *Nasonia* males is composed of stereotypic movements, which differ quantitatively and qualitatively between the species (see for details van den Assem and Werren 1994, Drapeau and Werren 1999, Beukeboom and van den Assem 2001 and Figure 2.3). Typically, courting males take up an invariable courtship position on top of the female, with the front legs placed on the female's head (Barrass 1960). A conspicuous feature of the courtship performance is head nodding: repeated bouts of nods that are separated by short pauses (Barrass 1960). The first head nod in a bout coincides with mouthpart extrusions, which is probably linked to the release of a pheromone (van den Assem et al. 1980). This pheromone is believed to involved in the process of induction of female receptivity (van den Assem et al. 1980), but its chemical composition has to date not been elucidated. If indeed the first head nods in a series coincide with pheromone release head nod frequency has at least a modulating role in the mating behaviour sequence. Therefore, head nodding behaviour and pheromone release are considered additional cues for the female to assess the male species.

A third factor that may play a role in *Nasonia* mate choice is male song. Male song, created by wing vibrations, is an important part of male courtship behaviour in *Drosophila*, where it has been studied in many species (e.g. Kyriacou and Hall 1982, Ritchie and Gleason 1995, Li et al. 2012). Male *Nasonia* wasps also vibrate their wings while performing headnods, and these songs are also seen as part of their courtship behaviour. These vibrations can be recorded (van den Assem 1986, Diao et al. unpublished) and potentially differ between the four species, for example by reflecting the differences in male wing lengths between the species. However, the functional significance of song patterns in the *Nasonia* mating sequence remains to be analysed and requires additional manipulative experimentation.

After assessing the above mentioned cues, females typically show their willingness to copulate, and thus acceptance, during the first head nod of a series by sweeping their antennae downwards and opening their genital orifice by raising their abdomen. Males immediately back up and copulation follows (Barrass 1960). Copulations last for 12-14 seconds and do not seem to differ between the species. Immediately after copulation the male performs a sequence of postcopulatory courtship before dismounting. If females do not become receptive, as manifested by their unwillingness to adopt the copulatory position, males will eventually terminate their courtship performance and dismount. When presented with an already mated female, males of the four *Nasonia* species differ in the number of headnod cycles they perform until dismounting. For example, *N. vitripennis* typically terminates courtship faster (after 7-8 cycles) than *N. longicornis* (after 12-13 cycles) (Beukeboom and van den Assem 2001). In addition, the number of cycles is affected by the number of courtship bouts that the male has performed in the recent past (Jachmann and van den Assem 1996).
The degree of prezygotic isolation between the *Nasonia* species is likely determined by an interaction between male courtship behaviour, chemical communication, male song on the one hand, and the females’ response to these traits, in terms of interspecific MD behaviour on the other. In contrast to males, that mate multiple times, females typically mate only once in nature, therefore selection for conspecific mating is expected to be stronger in females (van den Assem 1986, Burton-Chellew et al. 2007a, Grillenberger et al. 2008).

**Patterns of mate discrimination – no choice experiments**

Tests for MD usually consist of behavioural observation trails (or mating trails) in which successful copulations are scored as present or absent. Female MD is then defined as the percentage of females that rejected their mate. Mating trials in *Nasonia* are typically performed as no-choice experiments by placing one virgin male and one virgin female of standardized age (1 or 2 days old) in a small arena and observing them for 5-30 minutes. In intraspecific setups, the vast majority (>96%, Figure 2.4) of mating pairs result in copulations within the observation time frame. True choice experiments with two or more males or females are hard to perform as it remains difficult to interpret the interaction between interspecific male-male competition and female choice. The main reason for this may be that males of the different species differ strongly in their aggressiveness, with *N. vitripennis* males being the most aggressive (Leonard and Boake 2006, this chapter). By combining a number of no-choice experiment datasets that have been collected in our laboratories over the years, a general overview can be made of female MD patterns between the four *Nasonia* species (Figure 2.4). In these no-choice experiments, MD is scored when a male successfully mounts and courts a female, but the female does not signal receptivity and no copulation occurs. Although significant quantitative differences are found between the studies, this does not lead to qualitative differences in the observed patterns, and therefore the results of the different studies can be combined. Furthermore, results found in our study are quite similar to data shown in Bordenstein et al (2000). In the next section we will shortly discuss these similarities.

Strong asymmetric sexual isolation exists between the different *Nasonia* species, with *N. vitripennis* showing the highest female mate interspecific MD. *N. vitripennis* females have high MD against *N. giraulti* (90%) and *N. oneida* males (90%), but accept *N. longicornis* males more frequently (71% MD). *N. giraulti* and *N. longicornis* females show comparable heterospecific MD patterns against males of all three species: low MD against *N. vitripennis* males (20%), and slightly higher against *N. oneida* males (approximately 25%). As *N.*
Figure 2.4 General patterns of intraspecific and interspecific female MD in the four *Nasonia* species. For each species pair, the number of females is shown that rejected or accepted the male partner. Observations are performed using a variety of strains; the number of strains used per species is indicated below the female species name. Occurrence and strength of cytoplasmic incompatibilities, induced by species-specific *Wolbachia* infections, are shown for all species pairs.

*N. longicornis* and *N. giraulti* do not occur in sympatry (Darling and Werren 1990), MD between these two species is expected to be low. Our results are consistent with this, *N. longicornis* females hardly discriminate against *N. giraulti* males (8%), and neither do *N. giraulti* females against *N. longicornis* males (5%). Similarly, *N. oneida* females discriminate strongly (97%) against males of the sympatric species *N. vitripennis*, but MD against *N. longicornis*, which is allopatric to *N. oneida*, is lower (63%). *N. giraulti* males are more often accepted by *N. oneida*, with only 25% of the females rejecting the *N. giraulti* males. This last result contradicts the prediction that MD of species that occur in sympatry is higher than allopatric species pairs.

To further quantify the degree of prezygotic isolation between the four *Nasonia* species we calculated a prezygotic isolation index, using the following formula:

\[
i = 1 - \frac{\text{frequency of heterospecific matings of both reciprocal crosses}}{\text{frequency of homospecific matings of both species}} = 1 - \frac{[A \times B] + [B \times A]}{[A \times A] + [B \times B]} \tag{1}
\]
Indexes are shown in Table 2.1. We can infer that prezygotic isolation is strongest between *N. vitripennis* and *N. oneida*, and weakest between *N. giraulti* and *N. longicornis*, as is expected by their allopatric status. The prezygotic isolation index between *N. giraulti* and *N. oneida* is fairly low, contradicting what would be expected based on the theory of reproductive character displacement, which states that differences in behaviour (in this case displayed by MD) are stronger in sympatrically occurring species as opposed to allopatrically occurring species (Brown and Wilson 1956, Grant 1972).

Table 2.1 Patterns of prezygotic isolation between the four *Nasonia* species, as indicated by prezygotic isolation index (*i*). As this index is designed for heterospecific matings, no values can be calculated for intraspecific crosses. 0 = no isolation, 1 = complete isolation.

<table>
<thead>
<tr>
<th>species</th>
<th><em>N. vitripennis</em></th>
<th><em>N. longicornis</em></th>
<th><em>N. giraulti</em></th>
<th><em>N. oneida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. vitripennis</em></td>
<td>0.445</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. longicornis</em></td>
<td>0.553</td>
<td>0.040</td>
<td>0.204</td>
<td></td>
</tr>
<tr>
<td><em>N. giraulti</em></td>
<td>0.932</td>
<td>0.434</td>
<td>0.204</td>
<td></td>
</tr>
<tr>
<td><em>N. oneida</em></td>
<td></td>
<td></td>
<td>0.040</td>
<td>0.204</td>
</tr>
</tbody>
</table>

Interestingly, asymmetric patterns exist in MD. This means that for two species A and B, species A could strongly discriminate against species B, but species B might not necessarily show such strong MD against species A. To indicate asymmetric patterns of MD, the prezygotic isolation index was slightly adjusted:

\[
i' = 1 - \frac{\text{frequency of heterospecific matings with female of species A}}{\text{frequency of homospecific matings of species A}}. \tag{2}\]

Indexes for asymmetrical isolation are shown in Table 2.2. From these indexes it can be concluded that strong asymmetrical patterns of prezygotic isolations exist for crosses with *N. vitripennis* (except for *N. vitripennis* and *N. oneida*), reflecting the strong discriminatory behaviour of *N. vitripennis* females. Asymmetry was also found in the *N. longicornis* and *N. oneida* species pair, as a result of strong MD of *N. oneida* females. These results show that prezygotic isolation in the *Nasonia* complex is incomplete and confirm previous results (van den Assem and Werren 1994, Bordenstein et al. 2000). Additionally, the data are partially reflective of the geographical distribution and sympatry of the four *Nasonia* species, but are not entirely explained by these factors.
Table 2.2 Patterns of asymmetrical prezygotic isolation between the four Nasonia species, as indicated by the asymmetrical prezygotic isolation index ($i'$). The upper right section and lower left section can be compared to show asymmetries in isolation.

<table>
<thead>
<tr>
<th>Female species</th>
<th>Male species</th>
<th>N. vitripennis</th>
<th>N. longicornis</th>
<th>N. giraulti</th>
<th>N. oneida</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. vitripennis</td>
<td>N. longicornis</td>
<td>0.185</td>
<td>0.701</td>
<td>0.907</td>
<td>0.899</td>
</tr>
<tr>
<td>N. giraulti</td>
<td></td>
<td></td>
<td>0.021</td>
<td>0.059</td>
<td>0.262</td>
</tr>
<tr>
<td>N. oneida</td>
<td></td>
<td>0.965</td>
<td>0.609</td>
<td>0.792</td>
<td></td>
</tr>
</tbody>
</table>

In addition to these differences between the four Nasonia species, intraspecific differences in mating behaviour are common, and occasionally a N. vitripennis strain has been collected in Europe (e.g. ITA2 from Italy) that shows low interspecific MD. Also N. giraulti strains show large variations for interspecific MD against N. vitripennis (Table 2.3). Intraspecific crossings, however, are almost always successful regardless of the tested strains. For example, crosses between different N. vitripennis strains result in copulation in almost 100% of the cases. This has been tested by observing a total of 600 matings pairs of 20 strain combinations collected along a latitudinal gradient of 20 degrees (latitude 42-62) within Europe. Mate acceptance in was high (97%) between all strain combinations (data not shown), indicating that there is no isolation between the strains. Only significant differences in latency time were found (GLM, df=19, Chi=54.38, $p <<0.0001$). These results point at absence of intraspecific isolation within N. vitripennis strains. This is consistent with the observation that European and North-American N. vitripennis strains cross readily in the laboratory (see also the section on reinforcement below).

**Choice experiments – Implications of male-male competition**

In an experimental set-up in which we aimed to investigate female MD in N. vitripennis, when given a choice between a conspecific male and a N. giraulti male, we noticed a number of issues relating to male-male competition. A total of 200 N. vitripennis females were given a choice between a heterospecific and conspecific male. The conspecific N. vitripennis male was significantly more often the first male to court the N. vitripennis female (Chi-squared=39.8, df=3, $p<0.0001$). This shows that, when paired with a N. vitripennis female, N. vitripennis
Table 2.3 Variation between strains of the same species for interspecific mate discrimination (IMD) against *N. vitripennis* and *N. giraulti* males. Given is the sample size, percentage and standard error of females that discriminate against males of the other species.

<table>
<thead>
<tr>
<th>Female species</th>
<th>Male species</th>
<th>Total IMD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tbody>
<tr>
<td><em>N. vitripennis</em></td>
<td><em>N. vitripennis</em></td>
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</tr>
<tr>
<td><em>N. vitripennis</em></td>
<td><em>N. giraulti</em></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>N. giraulti</em></td>
<td><em>N. vitripennis</em></td>
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<tr>
<td><em>N. giraulti</em></td>
<td><em>N. giraulti</em></td>
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**Table 2.3** Variation between strains of the same species for interspecific mate discrimination (IMD) against *N. vitripennis* and *N. giraulti* males.
males almost always win the male-male competition with *N. giraulti* in terms of first mounting the female (Table 4). This is consistent with *N. vitripennis* males being more aggressive (Leonard and Boake 2006). Additionally, sneaking behaviour (van den Assem 1986) was observed in *N. vitripennis* males for 35% of the observed crosses, i.e. while the *N. giraulti* male performed courtship behaviour to induce female receptivity, the *N. vitripennis* male sits on the female’s abdomen and copulates with her upon becoming receptive. In a choice experiment with *N. giraulti* females paired with both a *N. vitripennis* and a conspecific male, *N. vitripennis* males showed sneaking behaviour in 54% of the observed crosses. In contrast, *N. giraulti* males never performed sneaking behaviour, in choice experiments with either a *N. vitripennis* or *N. giraulti* female (see Table 2.4). Sneaking behaviour appears to be a strategy that has evolved in response to strong male-male competition within *N. vitripennis*, which is supported by observations of sneaking attempts in intraspecific crosses (data not shown). As male-male competition and differences in aggression (for example sneaking attempts), between the *Nasonia* males of the different species, likely interact with female choice, it is ambiguous to make conclusions on female interspecific MD using this experimental set-up. Testing female MD in a true choice experiment will require an experimental set-up that does not allow for male-male interactions.

**Table 2.4** Mating behaviour differences in choice mating trials between *N. vitripennis* and *N. giraulti*.

<table>
<thead>
<tr>
<th>Species</th>
<th>First mounting of <em>N. vitripennis</em> ♂ in heterospecific contests.</th>
<th>Male sneaking behaviour in heterospecific contests.</th>
<th>Male sneaking behaviour in heterospecific contests.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. vitripennis</em></td>
<td>72% (N=200)</td>
<td>35% (N=192)</td>
<td>54% (N=94)</td>
</tr>
<tr>
<td><em>N. giraulti</em></td>
<td>58% (N=100)</td>
<td>0% (N=192)</td>
<td>0% (N=94)</td>
</tr>
</tbody>
</table>

**Genetics of Mate Discrimination**

The genetics of male reproductive behaviour in *Nasonia* has been investigated using interspecific crosses (Beukeboom and van den Asssen 2001; Beukeboom et al., in prep). These studies reveal a complex regulation with multiple QTL for different courtship components and abundant epistatic interactions between different loci. The genetic architecture of female mating behaviour has not been studied to the same extent (but see Velthuis et al. 2005). Recently, Giesbers et al. (Chapter 3, this thesis) analysed the genetic
basis of female interspecific MD in *N. vitripennis* against *N. giraulti* males using artificial selection. They found a fast and significant response to selection resulting in a 35% decrease in MD within four generations of selection. The cumulative heritability of interspecific MD in the artificial selection experiment was $h^2=0.29$, which shows the existence of substantial genetic variation for interspecific MD in *N. vitripennis*. Although more detailed genetic studies are required, these studies indicate that the *Nasonia* system is very well suited for investigating the genetic basis of female MD. Such studies can particularly be useful for testing the theoretically predicted genetic linkage between male signal and female choice traits, as well as for investigating the genetic basis of intraspecific versus interspecific mate choice.

**OTHER TRAITS INVOLVED IN PREZYGOTIC ISOLATION**

Another interesting prezygotic isolation inducing behaviour that has only been found in a few parasitic Hymenoptera species (da Costa Lima 1928, Suzuki and Hiiehata 1985, Drapeau and Werren 1999) is within-host-mating (WHM), in which males and females mate inside the host before emergence. The *Nasonia* species differ in their preference for WHM (Drapeau and Werren, 1999; This thesis, Chapter 5). For our experiments, WHM was scored by collecting all females from inside the host, at the moment when the first female has emerged from the host. Females that have mated inside the host will produce daughters and sons, while unmated females will only produce sons. Our results confirm earlier work by Drapeau and Werren (1999): *N. giraulti* has a high preference for WHM, while *N. vitripennis* almost always mates outside the host (Figure 2.5). *N. longicornis* and *N. oneida* show low levels of WHM. Drapeau and Werren (1999) already suggested that WHM might have evolved in *N. giraulti* to avoid interspecific hybridization. As a consequence of WHM, *N. giraulti* females will only encounter *N. vitripennis* males after having mated conspecifically within the host. As re-mating rates in natural populations of at least *N. vitripennis* are very low (van den Assem et al. 1980, van den Assem and Jachmann, 1999, Grillenberg et al. 2009b), this would produce a strong barrier against interspecific mating. Additionally, it might explain our observation that *N. giraulti* females discriminate less against *N. vitripennis* males than *N. vitripennis* females discriminate against *N. giraulti* males. Our results are generally consistent with the prediction that the rate of WHM is negatively correlated with the degree of interspecific MD. It remains to be seen if WHM is a male or female mediated trait, or the result of an interaction between both sexes.

In a study into the possible role of sexual conflict, Geuverink et al. (2009) compared copulation durations of conspecific and heterospecific crosses to test for possible mating incompatibilities (e.g. morphological or behavioural) between lines of *N. vitripennis* and *N. longicornis* that were cured from their *Wolbachia* infection. In both species, heterospecific crosses did not differ in duration from conspecific crosses which suggests that sperm
transfer is not mitigated in heterospecific crosses. This was further substantiated by occurrence of sperm in the spermatheca of heterospecifically mated females immediately after copulation. Moreover, the results indicated that the speed at which sperm is transferred and the amount of transferred sperm did not differ much between conspecific and heterospecific crosses. Furthermore, the survival of sperm in the course of time was not affected either, as females that had their reproductive period delayed by denying them access to hosts for the first ten days of their life, still appeared to have sperm in good condition in their spermatheca, regardless of whether they were heterospecifically or conspecifically mated. These data indicate that post-mating incompatibilities between heterospecific sperm and the spermatheca environment appear to play no role in *Nasonia*, at least between the two tested species *N. vitripennis* and *N. longicornis*.

Geuverink et al. (2009) used crosses between *N. vitripennis* and *N. giraulti* to determine whether female acceptance is influenced by the species of the first male partner. A clear effect of male species on female re-mating rate was found, both *N. vitripennis* and *N. giraulti* females mated more frequently after having first mated a heterospecific male. However, this increase was only significant for *N. giraulti* females, which may in part be due to the higher conspecific re-mating rate of *N. vitripennis* females under lab conditions. This shows

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**Figure 2.5** Interspecific differences in WHM. For all four *Nasonia* species, the proportion of females is shown that mated either inside or outside the host. Observations are performed using a variety of strains; the number of strains used per species is indicated below the female species name. Values in the pie slices indicate the number of females.
that heterospecific males are less efficient in decreasing female receptivity. At present it is not possible to differentiate between the mechanisms that could reduce receptivity, such as differences in male postcopulatory behaviour or physiological effects of sperm in the reproductive tract of the female. Interestingly, *N. giraulti* males have longer postcopulatory courtship than *N. vitripennis* males, which may reduce the re-mating rate of *giraulti* mated females.

**REINFORCEMENT**

Whether interspecific mating can lead to reinforcement of premating behaviour is still controversial, even though there is both theoretical support (e.g. Butlin 1987, Liou and Price 1994, Servedio and Noor 2003) and empirical evidence for its role in premating isolation (e.g. Blair 1955, Noor 1995, Saetre et al. 1997, Rundle and Schluter 1998, Jaenike et al. 2006). A prediction of the reinforcement hypothesis is that sympatric populations will have higher levels of MD than allopatric populations of the same taxa (Jaenike et al. 2006). We compared female MD of *N. vitripennis* strains occurring in microsympatry with *N. giraulti* (from North America) with that of allopatric strains (from Europe). The opposite comparison, involving MD of allopatric *N. giraulti* females towards *N. vitripennis* males, is not possible, because *N. giraulti* has to date not been found in areas where *N. vitripennis* is absent.

We used a no-choice experiment in which females of four allopatric (European) and four sympatric (North American) *N. vitripennis* strains were tested against males of an *N. giraulti* strain (North American) using crosses to males of their own strain as control. Females of the allopatric *N. vitripennis* strains accepted *N. giraulti* males in 2-26% of cases compared to 0-13% of cases of sympatric strains (Figure 2.6), which is not significantly different (GLMM: df=1, Chi=1.36, p=0.2441). The control experiments of the conspecific design showed an invariably high mating rate (near 100%) for both *N. vitripennis* and *N. giraulti*. Although female mate acceptance did not differ between the allopatric and sympatric strains of *N. vitripennis*, a trend exists towards longer duration of courtship bouts that *N. giraulti* males needed to induce receptivity in sympatric females compared to allopatric females (GLMM: df=1, Chi=3.47, p=0.063, Figure 2.7, only courtship bouts that resulted in receptive females were taken into account). This is in line with the prediction of the reinforcement theory. In nature, males may give up courting sooner and pursue another female, since more than one mating partner may be present on a patch. For latency time no significant difference between allopatric and sympatric females was found (GLMM:
df=1, Chi=2.87, p=0.0902, Figure 2.7), although there is a trend towards higher latency time in the sympatric design, in line with the reinforcement hypothesis.

Another prediction of the reinforcement hypothesis is that females that occur sympatrically will evolve reproductive character displacement resulting in stronger MD against males of their own species from allopatric populations, as for example found by

![Figure 2.6 Proportion of *N. vitripennis* females that mated in heterospecific (*N. giraulti* male, left panel) and conspecific (*N. vitripennis* male, right panel) no-choice experiments. Females from sympatric strains are indicated by grey bars; females from allopatric strains by black bars. The right most white bar represents the proportion mating females in a conspecific no-choice experiment with *N. giraulti* males. Sample sizes are shown between brackets on the x-axis. GLMM indicated no significant difference in proportion mating females between allopatric and sympatric strains in both heterospecific and conspecific no-choice experiments (Chi-squared=4.56, df=2, p=0.102, significance indicated by capital letters in the plot).
Figure 2.7 Box and Whiskers plots of latency time and courtship duration of five different designs in no-choice experiments. The two box and whiskers in the left panel show results of the heterospecific design with *N. vitripennis* (Vit) females from allopatric (Allo) and sympatric (Sym) strains. The three box and whiskers in the right panel refer to conspecific controls of both species. The two *N. giraulti* (Gir 1 and Gir 2) strains are pooled. There is no difference in latency time between allopatric (VitAllo x Gir) and sympatric (VitSym x Gir) heterospecific design. All conspecific designs differed significantly from the heterospecific designs. A pairwise comparison of courtship duration was significant for the heterospecific design, but not for the conspecific control design. Significance at $\alpha = 0.05$ level is indicated by capital letters in the plot. The top and bottom of the boxes give the 25th and 75th percentile; the line in the middle of the box is the median. The ends of the whiskers represent the 2nd and 98th percentile. Outliers are indicated by open circles. Sample sizes are given at the bottom of the plot. Statistical details are given in the text.
Jaenike et al. (2006) in two Drosophila species. We found no evidence supporting this prediction in our study as all conspecific designs, including those between females and males of different continents yielded high acceptance rates (over 96% successful matings in both directions). Furthermore, there was no significant difference in courtship duration within the conspecific design between allopatric and sympatric strains (GLMM: df=1, Chi =0.44, p=0.507, Figure 2.7). This indicates that the observed elevated courtship duration of the sympatric heterospecific design is not a strain effect, but may reflect early reinforcement. We conclude that reinforcement has at most slightly strengthened prezygotic isolation between these two species through courtship duration but not female mate acceptance.

Bordenstein et al. (2000) found that N. longicornis females discriminate stronger against N. vitripennis males than vice versa, which they believe is due to an increased exposure to hybridization of N. longicornis because its distribution range is embedded within that of N. vitripennis (see Figure 2.2). In addition, N. vitripennis is a generalist parasitoid and appears to have higher population densities than N. longicornis, which likely results in higher incidences of microsympathy. These authors also found some evidence for reinforcement within N. vitripennis as one allopatric strain of Minnesota showed higher receptivity to N. longicornis (88% mating) than sympatric females (40-66% mating). As reinforcement in N. giraulti may have acted upon the evolution of WHM, the strongest effect of reinforcement on female interspecific MD may actually be found in N. oneida. This species was only recognized as a separate species because of its strong MD against its sympatric congeners N. vitripennis and N. giraulti. No investigations of reinforcement have been performed with N. oneida as of yet.

One possible reason for not finding strong evidence of reinforcement in N. vitripennis is that not enough time has passed since its sympatry was established. Following results of van Opijnen et al. (2005), Raychoudhury et al. (2010b) found evidence from molecular studies that current N. vitripennis populations in North America result from a recent (re)immigration event from Europe. Thus, the courtship behaviour of N. vitripennis might not yet have adapted to encounters with N. giraulti. However, Giesbers et al. (Chapter 3, this thesis) have shown that interspecific MD of N. giraulti can decrease in response to artificial selection within a few generations. Other studies have also shown that traits that are important for mating success, such as courtship song in D. melanogaster, have relatively high heritability (Hedrick 1988, Ritchie and Kyriacou 1996). The temporal aspects of selection processes involving MD may differ considerably between laboratory settings and natural conditions. This requires further investigation of the behavioural interactions in microsympatric Nasonia populations. Finally, reinforcement of prezygotic isolation may actually have acted on different aspects of the mating behaviour in Nasonia than female MD, such as WHM (Drapeau and Werren 1999) or chemical communication (see below).
In conclusion, *Nasonia vitripennis* strains that occur in sympatry with *N. giraulti* seem to require longer courtship but otherwise do not show a strong increase in female MD against *N. giraulti* males. Reinforcement may rather have acted on other aspects of the mating system in *Nasonia*, e.g. WHM. The high aggression of *N. vitripennis* males, the tendency to be the first to mount the female in interspecific contests and the sneaking behaviour of *N. vitripennis* males potentially make heterospecific matings between *N. vitripennis* females and *N. giraulti* males rare in nature.

### CHEMICAL COMMUNICATION AND THEIR ROLE IN SPECIES DISCRIMINATION

Insects in general have exploited chemical signaling as their primary mode of communication (Greenfield 2002). Two main types of chemical cues and signals are commonly differentiated: long-range and close-range. (Thornhill and Alcock 1983, Blomquist and Vogt 2003, Guarino et al. 2008). Concerning the latter type, cuticular hydrocarbons (CHC) are particularly common as close-range cues in a wide variety of insect communication systems (Blomquist and Bagneres 2010). CHC are the prevalent fraction of the lipid layer on the outer segment of the insect cuticle (Lockey 1985, Howard and Blomquist 2005), and one of their main functions is the prevention of dessication (Hadley 1981). As for their role in insect communication, CHC have been shown to function as both major sexual (Simmons et al. 2003; Peterson et al. 2007; Oppelt et al. 2009) and interspecific recognition cues (Bagneres et al. 1991, Takahashi and Gassa 1995, Singer 1998). CHC have been exceptionally well studied in the fruit-fly genus *Drosophila* with respect to species-specific CHC variation and CHC-based mate and species discrimination (Buckley et al. 1997, Ferveur 1997, 2005). Concerning CHC studies in *Nasonia*, Carlson et al. (1999) were the first to investigate the CHC profile of *N. vitripennis* and found clear sex-specific differences between adult males and females. Furthermore, Steiner et al. (2006) found experimental evidence that female CHC profiles function as sex pheromones in *N. vitripennis*, initiating courtship behaviour in conspecific males. A comparative CHC study between two *Nasonia* species was done by Raychoudhury et al. (2010a), who established that CHC profiles were divergent enough to separate *N. oneida* from its most closely related sister species, *N. giraulti*, as well as distinguish between their sexes.

Buellesbach et al. (2013) extended the CHC profile comparison and analyzed qualitative and quantitative CHC differences between all four *Nasonia* species, and the respective sexes in each species. In this study, CHC were found to be sufficiently divergent to unambiguously separate the *Nasonia* genus according to sex and species (Buellesbach et al. submitted). Sex-specificity constitutes a general pre-requisite for CHC to function as cues in sexual communication, while species-specificity hints at their potential role in premating isolation. Pursuing this lead further, Buellesbach et al. (2013) also performed behavioural assays focussing on the species-specificity of female CHC as mating cues. Female CHC were
found to function as species-specific sexual cues for the males in most, but not all, *Nasonia* species.

![Figure 2.8](image.png)

*Figure 2.8* Courtship attempts of males from representative strains of all four *Nasonia* species towards conspecific and heterospecific female dummies in all 16 possible combinations. The number of courtship attempts in the respective conspecific combination was used as a reference against which the numbers of courtship attempts in the heterospecific combinations were tested for each respective male strain. Absolute numbers of courtship attempts for each male strain were tested with Bonferroni-corrected Fisher’s exact tests, significant differences (p < 0.0167) are indicated by different letters. 20 Trials were conducted in each combination. Strains used are: *N. longicornis* (IV7R2), *N. vitripennis* (NY07/18), *N. giraulti* (NGVA II), *N. oneida* (NONY 11/36).

We performed additional assays presented here to test for the significance of species-specific differences in female CHC profiles for assortative mating mediated by the males. In these assays, freeze-killed females were used as dummies to minimize other cues apart from CHC and those dummies were presented to con- and heterospecific males. We tested whether the males initiated courtship behaviour or not (Figure 2.8) and whether subsequent copulation attempts took place (Figure 2.9). The conspecific pairing was used as a reference for the heterospecific pairings. As a control, we accessed male courtship and copulation behaviour on freeze-killed female dummies from which the CHCs had been removed. No courtship or copulation attempts were initiated on these CHC-deprived female dummies at all (data not shown). This clearly hints at the significance of female CHC as sexual cues for males to initiate courtship and/or copulation attempts in *Nasonia*. Concerning courtship behaviour, a striking pattern of discrimination against heterospecific females most likely based on their CHC profile can be seen in *N. oneida* males,
who court conspecific female dummies significantly more than all investigated heterospecific female dummies (Figure 2.8). In contrast, *N. vitripennis* did not discriminate con- from heterospecific female dummies at all in courting (Figure 2.8). *N. longicornis* only showed pronounced discriminatory behaviour regarding the initiation of courtship against *N. oneida*. Unexpectedly, *N. giraulti* males were apparently more attracted to heterospecific female *N. vitripennis* CHC profiles than to their own conspecific female CHC profile when considering their courtship attempts (Figure 2.9). One possible explanation for this is that their habit of WHM has not resulted in any differentiation in chemical recognition.

![Figure 2.9](image)

**Figure 2.9** Copulation attempts of males from representative strains of all four *Nasonia* species towards conspecific and heterospecific female dummies in all 16 possible combinations. The number of copulation attempts in the respective conspecific combination was used as a reference against which the numbers of copulation attempts in the heterospecific combinations were tested for each respective male strain. Absolute numbers of copulation attempts for each male strain were tested with Bonferonni-corrected Fisher’s exact tests, significant differences (p < 0.0167) are indicated by different letters. 20 Trials were conducted in each combination. Strains used are: *N. longicornis* (IV7R2), *N. vitripennis* (NY07/18), *N. giraulti* (NGVA II), *N. oneida* (NONY 11/36).

These differences in attraction were even more pronounced when regarding the copulation attempts (Figure 2.9). *N. oneida* and *N. vitripennis* generally show the same pattern for copulation attempts as for courtship attempts (see Figure 2.8) with a pronounced discrimination in the latter and no discrimination at all against heterospecific female dummies in the former case, respectively. *N. longicornis* males show no significant differences in number of copulation attempts against heterospecific female dummies vs. conspecific dummies, although a trend towards more copulation attempts with conspecific
female dummies is apparent (see Figure 2.9). *N. giraulti* males constitute the most curious case, since they did not attempt to copulate with the freeze-killed female dummies at all, independent of whether they were con- or heterospecific (Figure 2.9). This indicates that female CHC profiles are not sufficient as sexual cues for *N. giraulti* males to initiate copulation.

Over all, our experiments indicate that CHC profiles are important as sexual cues for males of all investigated *Nasonia* species. However, their species-specificity in initiating both courtship and copulation attempts by the males greatly varies according to the investigated species pairings, from clear male discriminatory behaviour against heterospecific female profiles (as displayed in *N. oneida*) to none at all (as displayed *N. vitripennis*). *N. giraulti* males apparently require additional cues to discriminate con- from heterospecific females for both initiating courtship and copulation attempts on female dummies.

In conclusion, clear indications were found for a role of female CHC profiles in *Nasonia* as sexual cues potentially involved in prezygotic isolation, but further study is needed to shed more light on both the exact mechanism of female-mediated CHC signalling as well as the particular CHC compounds functioning as the major cues for the males. Taking a different angle to investigate the basis for species-specific CHC variation in *Nasonia*, Niehuis et al. (2011) investigated the genetic background of CHC differences between *N. vitripennis* and *N. giraulti*. Using hybrid males and taking advantage of their haplody, they found a large number of QTL (>100) governing species-specific CHC variation, distributed over all five chromosomes. The results revealed interesting associations to orthologue genes known to be involved in CHC biosynthesis in *Drosophila* as well as QTL hinting at hitherto unknown genes potentially involved in the biosynthesis of CHC compound classes (Niehuis et al. 2011). It will be an important next step to further analyze and unravel this unexpectedly complicated genetic architecture of CHC variation between the *Nasonia* species. This should ultimately lead to a better understanding of how CHC divergence is genetically maintained and biosynthetically governed.

Concerning non-CHC based, long-range chemical communication, Ruther et al (2007, 2008) were the first to isolate a male specific sex pheromone from *Nasonia vitripennis*. Males produce a mixture of (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolide (HDL) from glands in their abdomen which they deposit by abdomen dipping to attract virgin females. The pheromone can be classified as long-range as females perceive it at distances of up to 5 centimeters and it remains attractive for up to 2 hours (Steiner and Ruther 2009). HDL is only attractive to virgin females; after copulation females immediately become insensitive to it (Ruther et al. 2007). In a later study using sperm depleted males, Ruther et al. (2009)
could also show that virgin females’ olfactory responses were positively correlated with pheromone dose, proving the mate assessment function of HDL.

In addition to the HDL pheromone, males also release a short range aphrodisiac from their mandibular glands, which appears to be deposited directly onto the female antennae during their headnods in the courtship sequence. Van den Assem et al. (1980) were the first to show the existence of this aphrodisiac by sealing the male mouthparts with glue, resulting in females that did not become receptive upon being courted by manipulated males. Ruther et al. (2011) used the same technique to show that virgin females do not lose their attraction to HDL if they have not received this short range aphrodisiac, further supporting its role in inducing female receptivity. However, the exact chemical composition of this aphrodisiac remains elusive as it appears to be difficult to isolate and analyse, and decades of intensive research did not get any closer to its chemical detection and characterization so far (Ruther, Schmitt and Werren, pers. comm.).

CONCLUSIONS

We have presented and discussed the current knowledge of prezygotic hybridization barriers in the *Nasonia* species complex. Although it is evident that the strengths of these barriers vary, depending on the species pair considered, it is much harder to determine how these differences potentially have evolved. It is likely that most differences came into being after postzygotic reproductive isolation was established. The reason is that species-specific infections with *Wolbachia* bacteria causing cytoplasmic incompatibility between the different species are considered as the most likely cause of the speciation events in the *Nasonia* genus (Bordenstein et al. 2001). *N. oneida* appears to be a clear example of sympatric speciation as it originated within the distribution range of *N. vitripennis* and *N. giraulti*. Some geographic differentiation in MD within species is apparent in *N. vitripennis*, although lack of such evidence from the other species may be due to a lower intensity of investigation.

A conspicuous characteristic of the *Nasonia* genus is its strong differences in degrees of prezygotic isolation between species. These differences are often asymmetric in that females of one species are either more or less discriminative against males of the other species. The degrees of premating isolation are generally consistent with the divergence time of the species. However, while *N. oneida* is genetically very similar to *N. giraulti*, females show very strong MD against *N. giraulti*. WHM may constitute another mechanism to prevent interspecific matings in nature. Consistent with this notion, *N. giraulti* exhibits high levels of WHM in combination with low levels of interspecific MD.

Chemical communication is likely another important factor in prezygotic isolation between the *Nasonia* species. Chemical signals have been shown to play important roles in
the *Nasonia* mating patterns. Sexual cues and/or signals act on at least three different levels during the *Nasonia* mating procedure, i.e. the male-produced long-range pheromone, female CHC and the male mouthpart aphrodisiac. There is clear evidence from behavioural assays that at least one of these chemical cues or signals are not only involved in intraspecific sexual selection, but also play a role in interspecific sexual isolation. Differences in male courtship displays, and possibly courtship song, likely play a modulating role.

Differentiation in mate choice among strains within species suggests that considerable levels of genetic variation are present in natural populations. This notion is supported by high heritability of MD behaviour and a fast response to selection in the laboratory. There is, however, as yet no strong evidence for a role of reinforcement in the *Nasonia* species complex (Bordenstein et al. 2000, this chapter). Several explanations can be proposed, such as that the species have diverged too recently for reinforcement to become established, or that other behaviours or traits may have been selected to prevent hybridization (e.g. WHM, host choice). Although there is considerable overlap in the distribution ranges of the four species in North America, where species can even occur microsympatrically (e.g. in the same fly host), we still know little about behavioural interactions under natural conditions. Nevertheless, the tractability and genomic knowledge of the *Nasonia* genus makes it a promising system for further study of the evolution of prezygotic isolation and speciation.

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