Genetics of reproductive behaviour in Nasonia
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CHAPTER 6
Summarizing Discussion
Prezygotic isolation

Reproductive isolation plays a key role in the process of speciation (Coyne and Orr 2004). It generates genetic divergence between populations, and can lead to the emergence of new species. In many species pairs, for which isolation is incomplete, hybrid offspring have a fitness disadvantage (Mallet 1998). Therefore, selection is expected to reduce interspecific matings by favouring traits that eventually result in prezygotic isolation barriers (Dobzhansky 1937). Behavioural traits can play a large role in (maintaining) prezygotic isolation. However, while it is often easy to demonstrate differences in behaviour between species, determining which behavioural traits are responsible for establishing effective reproductive isolation between species proves to be a bigger challenge (Bakker and Pomiankowski, 1995). Additionally, still not much is known about the genetic basis of behavioural traits involved in prezygotic isolation (e.g. Arbuthnott 2009). Insight into the genetic architecture of such traits can give us information on how prezygotic isolation can evolve and at what speed speciation can be completed.

Parasitoid wasps of the *Nasonia* genus (Hymenoptera) occur in (micro)sympatry in North America. The four known species are postzygotically isolated through a species-specific *Wolbachia* infection, which partially or completely prevents the formation of hybrids depending on the species pair (Breeuwer and Werren 1990). Some species combinations are completely incompatible and do not yield any offspring, or only-male offspring, yet others are only partially incompatible and yield a reduced number of daughters (Bordenstein et al. 2001). These different levels of postzygotic isolation between the different species combinations are dependent on both the geographic location of the species and the fitness of their hybrid offspring. When postzygotic isolation is complete, it is expected that prezygotic isolation mechanisms evolve to prevent costly interspecific mating. In *Nasonia* we observe a range of strengths of postzygotic isolation, therefore it is expected that there are differences in strengths of prezygotic isolation mechanisms as well.

In the species combination *N. giraulti* and *N. oneida* prezygotic isolation seems to be the predominant barrier to hybridization, as their respective *Wolbachia* strains appear to be identical and they apparently do not show any postzygotic isolation (Raychoudhury et al. 2010a). Without any discriminating mechanisms to prevent these two species from mating, they would hybridize in nature and not be considered separate species. It is therefore expected that behavioural differences play a large role in the isolation between these two species.

In this thesis I investigated the reproductive behaviour of parasitoid wasps from the genus *Nasonia*, focusing on the genetic basis of two behavioural traits that are suggested to play a role in establishing prezygotic isolation between the four different species: female
interspecific mate discrimination (MD) and within-host-mating (WHM). I asked the following questions: What is the genetic basis of these traits and what is their inheritance pattern? Are there genetic correlations between these traits and other mating behaviour traits? Can female interspecific MD and WHM be considered efficient prezygotic isolation barriers?

**FEMALE INTERSPECIFIC MATE DISCRIMINATION**

To answer the question whether interspecific MD is an effective barrier to hybridization in this species complex, I performed a literature study and set up my own experiments to determine the amount of interspecific MD between all the species pairs. I found that, depending on the species pair considered, the strength of prezygotic isolation differed significantly between species pairs (Chapter 2). Prezygotic isolation (as calculated by the frequency of interspecific matings) was generally consistent with whether or not the species are occurring in sympatry and with the divergence time between the species pairs, i.e. prezygotic isolation is higher in sympatric species and older species pairs. The exception to this pattern are *N. oneida* females that show strong reluctancy to mate with *N. giraulti* males, despite them being phylogenetically very close (Raychoudhury et al. 2010a), which suggests that female MD has evolved fast in *N. oneida*. This result made this species pair an interesting subject for a QTL study on MD behaviour (Chapter 4).

It has been known for a long time that males of the different *Nasonia* species differ in courtship behaviour and pheromone composition (van den Assem and Werren 1994, Drapeau and Werren 1999, Ruther et al. 2007). However, the extent to which these male traits are being used by females as cues for interspecific MD has never been well established. Female *Nasonia* wasps can use a combination of cues perceived from the males to determine their willingness to mate. Male *Nasonia* wasps display courtship behaviour consisting of a typical series of components and movements. Even though this courtship behaviour is species specific, and could potentially be used by the female *Nasonia* wasps as a cue for interspecific MD, these differences between the species are apparently not sufficient to prevent interspecific mating (e.g. van den Assem and Werren 1994, Buellesbach et al. 2014, Chapter 2, this thesis). Female *Nasonia* can also use long-range chemical communication through male produced sex pheromones to assess the males she encounters (Ruther et al. 2007, 2008). Ruther et al. (2007) have shown that all *Nasonia* males produce the sex pheromone (4R,5S)-5-hydroxy-4-decanolide (RS-HDL) from glands in their abdomen, which they use to attract virgin females. *N. oneida* males are an exception, as they produce very little to no RS-HDL pheromone (Diao et al. submitted). Only *N. vitripennis* males produce an additional component, RR-HDL (4R,5R)-5-hydroxy-4-decanolide (Niehuis et al. 2013, Ruther et al. 2007). This additional component might be used by *N. vitripennis*
females to discriminate between heterospecific and conspecific males. \textit{N. giraulti} females, however show no response to the presence of this specific \textit{N. vitripennis} pheromone; they accept \textit{N. vitripennis} males readily and do not distinguish between \textit{N. giraulti} pheromone extracts and \textit{N. giraulti} extracts with added RR-HDL (Niehuis et al. 2013). In many insect species, females become reluctant to re-mating as a result of sex-peptides transferred in the male ejaculate during copulation (e.g. Wolfner 1997, Chapman 2001, Wedell 2005). In \textit{Nasonia}, however, the reluctance to re-mating is independent of the transfer of male ejaculate (Ruther et al. 2010). Instead, this change in female behaviour is caused in response to a male oral aphrodisiac that is deposited by the males during courtship (Ruther et al. 2010). Although van den Assem et al. (1980) were the first to show the existence of this aphrodisiac, its chemical composition has not yet been characterized.

Differentiation in female interspecific MD among strains, within species, suggests that considerable levels of genetic variation are present in natural populations (Chapter 2). This notion is supported by my finding of high heritability of MD behaviour and a fast response to selection for lowered interspecific MD in the laboratory. I showed that interspecific MD in \textit{N. vitripennis} females against \textit{N. giraulti} males can be artificially selected from 80% to approximately 50% within a few generations, a decrease of 30%. (Chapter 3). The fast response to selection suggests that low interspecific MD is a dominant trait, but I could not confirm this in crosses between the selected and non-selected lines. This finding is in agreement with artificial selection experiments for MD in \textit{Drosophila}, that demonstrated reduced interspecific MD in 12 generations (Izquierdo et al. 1992) and intraspecific MD in as few as 5-8 generations (Piñeiro et al. 1993; Singh and Chatterjee 1988). These studies indicate that behavioural isolation can evolve rapidly, as was previously suggested by Coyne and Orr (1997, 2004). However, a cautionary statement is at place here: in my experiment I have selected for a decrease in interspecific MD. Although this can give us information about the genes involved in interspecific MD and potentially prezygotic isolation, it does not necessarily tell us how the trait has evolved. The fast response I have found in my selection experiment implies that interspecific MD can decrease fast, but this does not necessarily mean it can also increase fast. It would be very interesting to set up selection experiments to increase interspecific MD. If these experiments show similar results, it would indicate that MD frequencies may still be selected to higher levels and perhaps eventually create a complete barrier to prevent interspecific matings in nature. As the only barrier to hybridization in the \textit{N. giraulti} – \textit{N. oneida} species pair appears to be prezygotic, this species pair would be an ideal candidate for an artificial selection experiment for increased interspecific MD.

The species combination \textit{N. giraulti} / \textit{N. oneida} formed the basis of the QTL study for MD against \textit{N. vitripennis} that I performed in Chapter 4. As these species are genetically the most similar \textit{Nasonia} species, it was expected that relatively few genetic changes had led to big
changes in the discriminatory behaviour of female *N. oneida*. To investigate the genetic basis of interspecific MD in these species, I set up reciprocal interspecific crosses between *N. giraulti* and *N. oneida* and scored interspecific MD frequencies in their hybrid offspring (Chapter 4). The observed patterns in female F1 interspecific hybrids and females from F3 clonal sibships indicated that *N. oneida* alleles, responsible for high MD against *N. vitripennis*, are dominant over *N. giraulti* alleles. In a quantitative trait loci (QTL) analysis I found several QTL of large effect which were located on different chromosomes. This indicated that interspecific MD against *N. vitripennis* males in the *N. oneida* – *N. giraulti* species pair has a polygenic basis. Sample sizes are very important in such QTL studies as too few numbers of recombinants analysed may result in a bias towards finding QTLs with major effect, and hamper the finding of minor QTLs (Beavis 1998; Xu 2010). To find these minor QTLs it is necessary that a QTL study uses a high number of recombinant individuals and also a high number of molecular markers. In my study I have analysed 412 recombinant F2 males for 27 molecular markers. This is sufficient to give a good view of major QTL of interspecific MD, however, for detecting minor QTLs this study could be improved by using more molecular markers, that are equally distributed over the chromosomes.

The rate at which a trait can evolve is largely dependent on that trait’s mode of inheritance. Therefore, to say more about the genetic architecture of interspecific MD, I tested for dominance of this trait in all my experiments. My selection for lowered interspecific MD indicates that the mode of inheritance for female interspecific MD is recessive (Chapter 3). *N. oneida* alleles, which cause high interspecific MD, inherit dominantly over *N. giraulti* as I showed in my QTL experiment (Chapter 4). In contrast, Velthuis et al. (2005) and Raychoudhury et al. (2010a) found a recessive nature of interspecific MD for *N. vitripennis* and *N. oneida*, respectively. Taken together these studies show that the mode of inheritance for MD is different depending on the species pair tested. This may mean that partially different traits are being used for MD between species pairs. In combination with the results of my selection experiment, which showed strong differences between replicates, it indicates that multiple modifier genes play a role that may act in a gene network. If this is the case, these co-adapted gene networks could potentially be disrupted in hybrid individuals, leading to contradicting and unpredictable results and transgressive phenotypes in hybrids.

In this thesis I made a step towards unravelling the genetics of interspecific MD in *N. vitripennis*, and in the species pair *N. giraulti* and *N. oneida*. Several additional studies are needed to further investigate the genetic basis of this complex trait. As a logical follow up of the selection experiment, one should look for genetic differences between the selected lines for lowered interspecific MD and the control lines. In fact, a RAD sequencing experiment, was already set-up (and results are currently under evaluation) to find single nucleotide polymorphs between the selected and control lines. Such genetic analyses will
give us insight into the genes that are responsible for this behaviour. Additionally, differences in behavioural phenotypes can be caused by allelic differences at genes, as can be detected with the QTL analysis, but they can also be the result of variation in gene expression. To gain more insight into the genetics of interspecific MD in *Nasonia* it is essential to study differences in gene expression for this trait as well, for example through micro-array analysis or RNA-Seq. When the genes for interspecific MD have been identified, a way to go forward is to determine their effects by genome editing, for example by knocking down the responsible genes and scoring the effect on discriminatory behaviour.

**WITHIN-HOST-MATING**

Within-host-mating (WHM) is a reproductive behaviour that has only been documented for a few parasitoid species (e.g. Dreyfus and Breuer 1944, Suzuki and Hiehata 1985). Females displaying this behaviour mate inside the host prior to emergence. Drapeau and Werren (1999) had previously shown that WHM is virtually absent in *N. vitripennis*, low in *N. longicornis* and high in *N. giraulti*. I confirmed these results with additional lines of *Nasonia*, I found absence or very low proportions of WHM of *N. vitripennis*, high WHM frequencies in *N. giraulti* and low frequencies in *N. longicornis* and demonstrated for the first time that *N. oneida* has a low frequency of WHM (Chapter 2). My results show that wasps freshly collected from the field, as well as hosts collected directly from the field, yielded similar results (for tested species *N. vitripennis* and *N. giraulti*), suggesting that WHM also occurs in nature for *N. giraulti*, and not solely in the laboratory as a result of, for example, different host species (Chapter 5).

*N. giraulti* is the only *Nasonia* species in which WHM has evolved to high frequency. This poses an interesting problem as *N. giraulti* and *N. oneida* are genetically the most related species. Either WHM must have evolved recently, after the split between *N. oneida* and *N. giraulti*, (Chapter 5), or WHM evolved in the *N. giraulti* – *N. oneida* ancestor and was secondarily lost in *N. oneida*. This last hypothesis would of course raise the question: if WHM acts as a barrier to interspecific mating with *N. vitripennis*, why would it be lost in *N. oneida* as this species also occurs micro-sympatrically with *N. vitripennis*?

Experiments that measured WHM frequencies in F1 hybrid offspring of interspecific crosses between several species pairs suggested that the behaviour is mediated by two effects: (1) an effect of the females present in host. This is a genetic component that is dependent on both parental species, based on the finding that hybrid offspring show intermediate patterns of WHM; and (2) an effect of male species present in the host. Previously, it was suggested that WHM is a female trait and that this behaviour could help
to prevent interspecific matings with aggressive *N. vitripennis* males (Drapeau andWerren 1999, Leonard and Boake 2007). However, the results of my multiparasitization experiments contradict this hypothesis. In *N. giraulti*, the presence of an artificially created exit hole significantly decreased WHM and let to earlier emergence of the *N. giraulti* females. This indicates that the timing of the creation of exit holes (made by the males) is an important factor in manifesting WHM behaviour, suggesting that WHM is not a female trait, but a male-mediated trait. Most importantly, my results demonstrate that *N. giraulti* no longer mate inside the host when *N. vitripennis* is also present in that host - because *N. vitripennis* males create an exit hole immediately after their eclosion, resulting in advanced emergence of *N. giraulti* females - which leads to the conclusion that WHM is not an efficient prezygotic isolation mechanism.

My results raise a number of questions regarding the evolution of WHM behaviour: To what extent is this behaviour adaptive? Do *N. giraulti* males purposely wait before chewing a hole in the host puparium wall, to enhance their reproductive success by mating females before emergence? Or has WHM evolved as a by-product of the constraint that male *N. giraulti* cannot create an exit hole timely, for example, because of changes in their mandible morphology making it difficult or impossible to do so. It would be very interesting to devise an experiment in which one can actually observe what is happening inside the host after the males and females have eclosed from their pupal skin, using hosts parasitized by a single *N. giraulti* female as well as hosts multiparasitized by both *N. giraulti* and *N. vitripennis* males. Additionally, it would be interesting to investigate whether WHM can play a role in preventing interspecific matings between *N. giraulti* females and *N. oneida* males. As *N. oneida* males do not develop as fast as *N. vitripennis* males, they might not create the exit hole to allow the *N. giraulti* females to emerge. Alternatively, one might consider WHM as simply evolved as a result of drift.

**GENETIC CORRELATIONS**

If traits are genetically correlated it can influence the rate at which such traits can evolve. For traits involved in prezygotic isolation this is important as it can determine the rate at which speciation can take place (Coyne and Orr 2004). As an addition to my artificial selection experiment for lowered interspecific MD against *N. giraulti*, I scored the selected and the control lines for correlated responses in four reproductive behaviours. I found evidence for genetic correlations for interspecific MD and intraspecific MD, but not for interspecific MD with female re-mating and WHM. This suggests that interspecific MD and WHM are not controlled by the same genetic architecture, nor that there is a trade-off between the two. Decreased interspecific MD against males of one species also resulted in decreased interspecific MD against males of another species, suggesting that interspecific
MD in *N. vitripennis* is a “general” discriminatory trait with a similar, or at least partially shared, genetic basis for MD against other *Nasonia* species. This could work, for example, when *N. vitripennis* females use a cue that is specific for *N. vitripennis* males and not present in the males of the other species. When the received cue is not a *N. vitripennis* cue, i.e. any other *Nasonia* species male, the *N. vitripennis* female rejects the male. Alternatively, as intraspecific mating was faster for the females that were selected to accept heterospecific males, my results could also be explained by a general decrease in receptivity threshold (choosiness) in the selected females. It might be that a female will accept a male if the male cues meet a certain threshold. If the female becomes less choosy, i.e. lowers her selection criteria, she would accept males faster, regardless of the species of the male.

Additionally, I found that selection for decreased interspecific MD also leads to faster intraspecific mating, suggesting that there is strong genetic correlation between interspecific and intraspecific MD in *N. vitripennis*. I argue that selection for a decrease in prezygotic reproductive isolation may lead to a decrease in the strength of (intraspecific) sexual selection as well. This correlation may have important consequences for the evolution of prezygotic isolation barriers. Prezygotic isolation is expected to become stronger as the species divergence time increases (Coyne and Orr 2004) and therefore this correlation could imply that when the behavioural isolation between two related species increases, intraspecific sexual selection within those two species also increases. This last hypothesis was also proposed (in respect to a decrease in prezygotic isolation) by Carracedo et al. (1987), who suggested that for females with a high tendency to hybridize, sexual selection favouring high receptivity could be counteracted.

The results from my selection experiment raise a very interesting question: what exactly did I select for in these lines to lead to these results? Obviously this is speculation, but it is very likely that my selection has acted on a female receptor that can assess species specific male traits. The fast response to selection for lowered interspecific MD suggests that this female receptor has weakened or the threshold for that receptor was lowered. A single mutation could cause a receptor to become non-functional, leading to lower MD, but more likely this is modulated by changes in gene expression. So far, the receptor genes have hardly been studied (except see Robertson et al. 2010), and nothing is known about the genetic architecture of the female receptor traits involved in analysing male cues. My selected lines could help in this respect, for example by testing the females in their responses to male pheromones of the different species. Additionally, when analysing the genes that were selected, we might be able to identify the receptor genes that play a role in interspecific MD.
PREZYGOTIC ISOLATION IN NASONIA

As postzygotic isolation is near complete in all Nasonia species pairs, with the exception of the sympatric N. giraulti / N. oneida and allopatric N. giraulti / N. longicornis, it is expected that prezygotic isolation mechanism have evolved (or are evolving) to prevent costly interspecific mating. In this thesis I have shown that female interspecific MD and WHM are not sufficient alone to prevent interspecific mating. I believe it would be very worthwhile to look for other behavioural and/or morphological traits that could be involved in prezygotic isolation in Nasonia. Additionally, the male traits which can be used by the females as cues (e.g. the production of cuticular hydrocarbons, pheromones, male song), but also male behaviour such as male MD and male aggression will need to be investigated for all Nasonia species in choice experiments, preferably using multiple species in one test. While our experiments have shown that prezygotic isolation in the Nasonia complex is incomplete with regard to female interspecific MD and WHM, they do not tell us much about the natural situation. Little is known about the mating interactions in nature. Perhaps an interplay between the abovementioned behaviours and yet undetected communication ways and/or differences in morphology lead to complete prezygotic isolation. To fully understand the mating interactions in the Nasonia complex requires measuring of combined behaviours in all species (preferably both in the lab and in the field), between all species combinations, and in interspecific hybrids, as well as artificial selection experiments and manipulation experiments. I think that there is a need for more field experiments. All results, from choice and no-choice experiments in Nasonia are obtained through laboratory experiments. How can we draw any conclusions about prezygotic isolation in a species complex when we know so little about their behaviour in nature? While I completely acknowledge the difficulties that arise when doing field experiments, I think this might be partially circumvented by designing laboratory environments that are more natural for the wasps. For example, one could use bigger test arenas (instead of tiny tubes), larger numbers of individuals, and also different combinations of species’ males and females. Perhaps there are complex interactions that cause prezygotic isolation in this species complex, which can only be seen under nature-mimicking conditions.

We also need a better understanding of the role of chemical communication in the Nasonia mating interactions. A number of studies have investigated the long-range chemical communication in Nasonia, through male produced sex pheromones (e.g. Ruther et al. 2007, 2008, 2010, Niehuis et al. 2013). Female Nasonia were shown to assess males by their these long-range sex pheromones produced by male Nasonia except for N. oneida (Ruther et al. 2007, 2008, Diao et al. submitted). The fact that N. oneida does not produce much of this long range pheromone makes one wonder how partner attraction occurs in
this species. The role of chemical communication in interactions between *N. giraulti* and *N. oneida* clearly needs further investigation. Moreover, *N. vitripennis* males produce an additional sex pheromone component (RR-HDL) (Niehuis et al. 2013, Ruther et al. 2007). Whereas *N. giraulti* females do not respond to this component, the responses of *N. longicornis* and *N. oneida* to RR-HDL have not been investigated. I believe that the study of male pheromones in *Nasonia* will give us more insight into the mating interactions and prezygotic isolation in this species complex.

Another component that might be involved in behavioural isolation in *Nasonia*, is the short range aphrodisiac that males produce during courtship (van den Assem et al. 1980). This component is released during the head nods that males perform as part of their courtship display. It may act in interaction with other behaviours and or pheromones to create a barrier to hybridization. Future studies should focus on characterizing this component and comparing the presence and role of this aphrodisiac in all *Nasonia* species. Ultimately, it should be considered in combination with all previously scored traits that potentially play a role in prezygotic isolation.

Another behaviour that is rarely studied in *Nasonia*, but might play a role in behavioural isolation, is male MD. Buellesbach et al. (2014) showed that, although male *N. vitripennis* do not appear to discriminate against females of the other *Nasonia* species, the other species do show some reluctance in courting heterospecific females. In nature, selection may act on male MD in addition to female MD and generate a stronger barrier to hybridization. Therefore, it is also interesting to look at female traits that potentially serve as cue for male MD. One of these female traits are cuticular hydrocarbons (CHCs) that are present on the outer surface of females. These CHCs are known to serve two functions in insects, protection from desiccation and chemical communication (Hadley 1981, Blomquist and Bagnères 2010). They are species-specific and it was shown that *Nasonia* males can perceive and use them as a cue for MD (Steiner et al. 2006, Buellesbach et al. 2013). Recent studies showed that CHCs can play a role in both mate choice and ecological divergence, and it has been suggested that the interaction between these roles can influence the evolution of reproductive isolation (Chung and Carroll 2015).

Even though the cost to mating heterospecific individuals is much higher in females compared to males, male MD could still play a role in combination with other traits to prevent interspecific mating. I suggest that all above described traits: female interspecific MD, the production of sex pheromones and aphrodisiac in males, the production of CHCs in females, male MD and other behaviours which we so far have not been able to find, should be investigated for all species, in all species’ combinations. This way, we can establish the precise role of behavioural and chemical communication in the mating interactions in *Nasonia* and obtain a complete picture of how prezygotic isolation is manifested.
THE *N. oneida* - *N. giraulti* SPECIES PAIR

*N. oneida* and *N. giraulti* share a recent common ancestor and are distinguished as separate species because of strong behavioural isolation (Raychoudhury 2010a). My data suggest that the recent origin of *N. oneida*, within the distribution range of *N. giraulti*, may have been facilitated by the evolution of a few dominant alleles for interspecific MD. Moreover, a small genetic change within *N. giraulti* could have led to the evolution of WHM in this species. I believe that this species pair makes an ideal system to further study the genes involved in prezygotic isolation in *Nasonia*, as there appears to be no postzygotic isolation between these species (Raychoudhury et al. 2010a). As we have not been able to find reproductive isolation through interspecific MD in this species pair it means that another (behavioural) trait must be responsible for preventing the hybridization of these species. It would be a great opportunity to look for that trait in this species pair. A first logical step would be to investigate the effects of cuticular hydrocarbon compounds of males and females of these species on behavioural isolation, as these are clearly different for both sexes and species (Raychoudhury et al. 2010a). Another suggestion would be to investigate the importance of WHM in this species pair. What happens when a host is multiparasitized by a *N. giraulti* and a *N. oneida* female? Does the frequency of WHM decrease, as observed in the combination of *N. giraulti* and *N. vitripennis*? Additionally, studying behaviours such as host preference, male aggression, re-mating and dispersal in this relatively young species pair will provide a good starting point for unravelling the isolation barriers that prevent these two *Nasonia* species from hybridizing in nature.