Genetic architecture of prezygotic reproductive isolation in the parasitic wasp genus Nasonia
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Chapter 7 General discussion and conclusions

Wenwen Diao
PREZYGOTIC ISOLATION

One important question in speciation biology is how reproductive isolation barriers become established, which generally includes prezygotic and postzygotic isolation barriers that act before and after fertilization (Orr and Presgraves 2000; Presgraves 2010). Prezygotic isolation appears to evolve at lower levels of overall genetic divergence than postzygotic isolation, at least in sympathy (Coyne and Orr 1989). A reason for this may be that mating behaviour differences often form primary reproductive barriers and evolve rapidly through sexual selection in the early stage of the speciation process (Grant and Grant 1997). Therefore, genetics of behaviour is an important field of research for understanding the speciation process.

Our knowledge of the genetic basis of traits responsible for reproductive isolation is still rudimentary. Unresolved issues include whether the initial establishment of reproductive isolation is due to a few genes with large effect or many genes with small effect, with strong selection for a few traits or weaker selection on multiple traits, whether divergence relies on standing genetic variation or new mutations, and whether most genetic changes cause reproductive isolation or only appear following the speciation event (Barton and Gale 1993; Rice and Hostert 1993; Nosil 2008; Marie Curie SPECIATION network 2012). With recent genomic technologies (e.g., SNPs and candidate genes), we are now having more capability to explore the genomic perspective of speciation (Nosil and Feder 2012). Some “speciation genes” causing postzygotic isolation have been identified (Presgraves and Glor 2010), but less is known about the genetic basis of traits responsible for prezygotic isolation (Arbuthnott 2009). Insight into the genetic architecture of prezygotic isolation traits that drive divergence, such as mating behaviour, can provide information on how prezygotic reproductive isolation evolves as part of the process of speciation.

A model system for genetic study of reproductive isolation barriers is the *Nasonia* genus (Hymenoptera, Pteromalidae). *Nasonia* are parasitoid wasps that can be found in bird nests and on carcasses (Whiting 1967; Pultz and Leaf 2003; Grillenberger et al. 2008). The *Nasonia* genus consists of four closely related species, of which three species occur only in North America (including *N. longicornis*, *N. giraulti* and the more recently discovered *N. oneida*) and one cosmopolitan species (*N. vitripennis*) (Walker 1836; Darling and Werren 1990; Raychoudhury et al. 2010a). The four species differ in degrees of prezygotic and postzygotic isolation (Raychoudhury et al. 2010a; Giesbers et al. 2013). Differences in mating behaviour and chemical communication signals appear to play a role in premating isolation between *Nasonia* species. The *Nasonia* male releases sex pheromones, performs courtship behaviours and produces sounds by vibrating its wings to induce female receptivity and copulation (van den Assem et al. 1980; van den Assem 1986; Ruther et al. 2009; Nagele and Granato 2013; Giesbers et al. 2013).
2007, 2008; Clark et al. 2010; this thesis). How these different traits are involved in prezygotic isolation of species pairs is however not yet clear.

In this thesis, I investigated phenotypic variation in traits that appear to be involved in prezygotic reproductive isolation in the genus Nasonia. I focused on mating behaviour (including courtship behaviour and courtship song) and chemical communication signals (including sex pheromones and cuticular hydrocarbons). I asked the following questions: What is the variation in potential prezygotic isolation traits within and between species? Is there any geographical pattern in those traits within a species? To obtain a better understanding of the genetic basis of mating behaviour and sex pheromones, that are suggested to play a role in establishing prezygotic isolation between the Nasonia species, I performed a QTL study combined with SNPs markers in candidate genes. The knowledge obtained of the phenotypic variation and the genetic basis of reproductive isolation in this model system contributes to more insight in the speciation process.

MATING SIGNALS IN PREZYGOTIC ISOLATION

Mating behaviour

Courtship behaviour is known to play a pivotal role in the onset of population divergence leading to premating reproductive isolation between species as part of the process of speciation (e.g., Williams et al. 2001; Hoikkala et al. 2005; Arbuthnott 2009). Male courtship and mating behaviour of three of the Nasonia species, N. vitripennis, N. longicornis and N. giraulti, have been well described (Cousin 1933; Barrass 1960a, 1960b, 1961; Whiting 1967; van den Assem 1986; van den Assem and Werren 1994; van den Assem and Beukeboom 2004; Peire Morais 2007), but not for the recently discovered species N. oneida. Courtship displays of these three species are sufficiently diverse to be diagnostic at the species level, both on qualitative differences (species-characteristic motor patterns) and quantitative differences (number and/or duration of similar elements) (van den Assem and Beukeboom 2004). To complete the inventory in the Nasonia genus, I described the male courtship and mating behaviour of the youngest species in this genus, N. oneida, and compared it to the other three Nasonia species (Chapter 2). I found that N. oneida displays the same basic courtship and mating behaviour pattern as the other three Nasonia species, but it can also be recognized by its species-specific characteristics with more resemblance to N. giraulti than the other species. This result is consistent with the divergence time between the species within the Nasonia genus (Campbell et al. 1993; Raychoudhury et al. 2010a).

N. oneida is considered a separate species based on its strong behavioural isolation from the other Nasonia species (Raychoudhury et al. 2010a). In particular, N. oneida females discriminate strongly against N. giraulti males, whereas N. giraulti females are less
discriminatory towards *N. oneida* males (Raychoudhury et al. 2010a; Giesbers et al. 2013; Chapter 6, this thesis). There is little or no postzygotic isolation between *N. oneida* and *N. giraulti* (Raychoudhury et al. 2010a). As *N. oneida* is sympatric with *N. vitripennis* and *N. giraulti* in its native range in New York State (Raychoudhury et al. 2010a), this high level of mate discrimination probably has a role in maintaining genetic integrity of the species in nature. The *N. giraulti – N. oneida* species pair is therefore a very good combination for research into the underlying genetic basis of prezygotic isolation.

**Male courtship song**

Another behavioural factor that can play an important role in insect communication and the mating ritual is the male courtship song. Courtship song as an acoustic component has been shown to be an important part of male courtship display and mate choice in many species, such as crickets and *Drosophila* (e.g., Kyriacou et al. 1990; Ritchie and Kyriacou 1994; Ritchie et al. 1999; Montealegre-Z et al. 2011; Debelle et al. 2014). Courtship songs in *Drosophila* have been extensively analysed (e.g., Cowling and Burnet 1981; Robertson 1983; Cobb et al. 1989; Ritchie and Kyriacou 1994). For instance, some species of *Drosophila* were shown to produce two types of song, which are pulse song and sine song (Shorey 1962; von Schilcher 1976). Courtship song differences were reported to contribute to reproductive isolation between species in *Drosophila*. For instance, Ritchie et al. (1999) found that females mated most quickly when stimulated by song typical of their own species. Some of the song parameters were shown to be important for species recognition in *Drosophila montana*, such as inter-pulse interval (Ritchie et al. 1998), and song cycles were claimed to be important for mate discrimination (Kyriacou and Hall 1980, 1989; Crossley 1988; Ewing 1988; Ritchie et al. 1999). Like *Drosophila*, male *Nasonia* also produce sounds during courtship by vibrating their wings (van den Assem and Putters 1980; van den Assem and Beukeboom 2004). Previous studies have described the courtship song of *N. vitripennis* in some detail (van den Assem and Putters 1980), but no studies have been done on the other three *Nasonia* species.

I provided detailed descriptions of male courtship song for all four *Nasonia* species (Chapter 4). My results indicated that all *Nasonia* species share a clear hum song that is comparable to the sine song of some *Drosophila* species, but it lacks the pulse song of *Drosophila*. Hence, song parameters used to describe the pulse song in *Drosophila*, such as the number of pulses and interpulse-interval (Lagisz et al. 2012), are not relevant to *Nasonia*. The male courtship songs of the four *Nasonia* species are similar in general structure with song bouts consisting of a series of bursts interrupted by pauses, but they differ on specific aspects including duration and number of bursts, pause duration between bursts and frequency (Chapter 4). Additionally, my results revealed that the overall song
patterns of *Nasonia* can be linked to their behavioural courtship components. For instance, the song bout is synchronous with one behavioural cycle, consisting of a series of headnods followed by a pause in male courtship display (van den Assem 1986; van den Assem and Beukeboom 2004). The species-specific quantitative differences in song characteristics of *Nasonia* (Chapter 4) are analogous to the differences in behavioural components of courtship between the species (Chapter 2). Although I have found male courtship song differences in the four *Nasonia* species, we do not yet know the genetic basis of song differences between the *Nasonia* species and how important the song differences are for inducing female receptivity in *Nasonia*. Further experimentation, such as Quantitative Trait Locus (QTL) analysis and experimentally manipulating song patterns (e.g., silenced males with playback song) is required to test whether song differences play a major role in sexual isolation between the species.

Wing size can have an effect on courtship song production in *Drosophila* (Ewing and Bennet-Clark 1968; Hoikkala and Aspi 1993). In *Nasonia*, van den Assem and Putters (1980) have investigated the role of wing size on song frequency in *N. vitripennis*, and found no effect. My results (Chapter 4) showed that *Nasonia* species with smaller wings (*N. vitripennis* and *N. longicornis*) had less structured courtship song than species with larger wings (*N. giraulti* and *N. oneida*). *Nasonia* species with shorter wings produced shorter burst length, shorter pause duration between bursts/burst series, higher number of bursts per bout, and different song frequency. Therefore, wing size could be one of the factors that affect male courtship song differences between *Nasonia* species. Further investigation is needed about the significance of wing morphology on courtship song in the *Nasonia* genus. This could, for instance, be done by experimental removal of the wings, or by gluing them together. As the genetic architecture of wing morphology is well known in *Nasonia* (Loehlin *et al.* 2010a, 2010b) another possibility may be to introgress wing genes from one species to another.

**Chemical signals - sex pheromones and cuticular hydrocarbons**

We also need to understand chemical communication signals, which are probably the oldest means of communication in insects, and might be involved in prezygotic isolation as well. There are two general classes of chemical signals, one is volatile pheromones such as sex pheromones, and the other is non-volatile pheromones such as cuticular hydrocarbons (CHCs). Sex pheromones are often produced by one of the two sexes as a specific odour that is recognized by the other sex leading to attraction and copulation between the partners. CHCs are present on the body surface of insects. They can serve multiple functions, such as protection against desiccation, a barrier against microbial infection, species recognition cues, and can also act as chemical cues in mate choice (Suvanto *et al.*
CHCs can only function as sex pheromones when they show sexual dimorphism allowing for recognition of conspecifics or mating partners. In *Nasonia*, the male produces a long-range sex pheromone from a gland in its abdomen to attract the female from a distance (van den Assem et al. 1980; Ruther et al. 2007, 2008). This pheromone likely plays a role in the duration of the latency time (i.e., the time from introducing a male to a female and the moment of the male mounting the female) (Diao et al. 2016; Chapter 6, this thesis). After a latency period, the male recognizes the cuticular hydrocarbon profile of the female and mounts the female (Buellesbach et al. 2013) to start his courtship display. During his display, the male deposits an as yet unidentified short-range pheromone (aphrodisiac) by mandible contact with the female’s antennae to induce receptivity in courted females (van den Assem et al. 1980; Ruther et al. 2010).

Two long-range sex pheromones can be produced by *N. longicornis*, *N. giraulti* and *N. oneida* males: RS-HDL ((4R,5S)-5-hydroxy-4-decanolide) and 4-methylquinazoline (Niehuis et al. 2013; Diao et al. 2016). *N. giraulti* males were found to produce large amounts of RS-HDL and 4-methylquinazoline (Niehuis et al. 2013; Ruther et al. 2014; Diao et al. 2016; Chapter 6, this thesis). I further found that *N. oneida* males produce the RS-HDL at an almost 10-fold lower level than *N. giraulti* males (Chapter 6). This difference in sex pheromone quantity of *N. giraulti* and *N. oneida* (Chapter 6) raises an interesting question: how does mate attraction occur within the *N. oneida* species without the long-range male sex pheromone RS-HDL? It seems that the lower quantity of RS-HDL in *N. oneida* does not negatively affect their acceptance by *N. giraulti* females. In contrast, the high quantity of RS-HDL in *N. giraulti* males appears to function as a repellent to *N. oneida* females. This explanation is consistent with rapid shifts in sender and receiver cues in establishing prezygotic isolation in this genus (Buellesbach et al. 2013; Niehuis et al. 2013). The role of the RS-HDL pheromone in the mating process of the various *Nasonia* species deserves further attention.

CHCs are long-chain molecules that are synthesized in specialized cells and subsequently transported to the cuticle of an arthropod, which mainly consist of three substance classes: alkanes, alkenes, and methyl-branched alkanes. Genes for alkene biosynthesis have been identified in a QTL study of CHC differences between *N. giraulti* and *N. vitripennis*, with a high similarity to *Drosophila* (Niehuis et al. 2011). CHCs appear to perform different functions in females and males regarding prezygotic isolation. Strong divergence in *Nasonia* CHC profiles between species and sexes have been found (Carlson et al. 1999; Raychoudhury et al. 2010a; Buellesbach et al. 2013; Chapter 5, this thesis). Female CHCs normally act as close-range sex pheromones (Steiner et al. 2006), and are used for species and mate recognition by males, with the exception of *N. giraulti* where it appears
that the pheromonal function has been lost (Buellesbach et al. 2013). For male CHCs, no sexual signalling function could be found so far (Steiner et al. 2006; Buellesbach et al. 2013; Giesbers et al. 2013). It appears likely that the sex pheromone function of female CHCs is an ancestral state in the *Nasonia* genus. Divergent selection for mate signals is therefore expected to be less strong for females than males, and female CHC adaptation to geoclimatic factors may be less constrained by sexual selection.

Additionally, it has been known that CHCs are not invariant and static traits, but can co-vary with environmental and geoclimatic factors (Ingleby 2015). The effect of environmental factors on CHCs has been shown in several species, *e.g.*, beetles (Toolson and Hadley 1979), grasshoppers (Rourke 2000), *Drosophila* (Rouault et al. 2000) and ants (Menzel et al. 2017). However, information on the evolutionary impact and possible adaptive significance of geoclimatic factors on intraspecific CHC composition is still scarce (Rouault et al. 2000). No study so far has considered a potential influence of geoclimatic factors on intraspecific CHC composition in *Nasonia*. In this thesis, I made a first step towards identifying geoclimatic effects on intraspecific CHCs composition in the *Nasonia* genus (Chapter 5). I performed gas chromatography/mass spectrometry (GC/MS) analyses to describe CHC profiles from seven European sample localizations and climatic data obtained from publically available databases. I correlated different CHC compound classes (including alkanes, alkenes, monomethyl-alkanes, dimethyl-alkanes, and trimethyl-alkanes) with several geoclimatic factors (latitude, mean temperature, temperature range, humidity and precipitation). I focused on alkanes relative to the other compound classes as alkanes are known to be the most important substance class for hydrocarbon layer hardening (Gibbs and Pomonis 1995; Gibbs 1998, 2002). My results revealed significant correlations between the CHC ratio (alkanes - monomethyl-alkanes) / (alkanes + monomethyl-alkanes) with latitude, mean temperature, and temperature range among females; and also between CHC ratios in females, but not in males, with mean annual precipitation rates (Chapter 5). This indicated that geoclimatic factors, in particular latitude, temperature and precipitation, do affect CHC composition in *Nasonia*. I concluded that female CHCs are more conducive to environmental influences than male CHCs (Chapter 5).

Selective pressures exerted by geoclimatic factors have been hypothesized to influence biosynthesis and genetic background of CHC compounds (Rouault et al. 2000, 2004; Frentiu and Chenoweth 2010). For instance, increase of the hardening of the CHC profile can better protect against dessication, which is suggested to be the ancestral function of CHCs in insects (Gibbs 2002). CHCs with better protection against desiccation are therefore expected to be produced in warmer and dryer climates. There are several ways to improve the hardening of the CHC profiles. One is to increase the chain-length of compounds even within one substance class, as reported by Rouault et al. (2000). My study showed an
increase of alkanes compared with other substance classes as an alternative way to increase the hardening of CHC profiles (Chapter 5). The results of Rouault et al. (2000), Frentiu and Chenoweth (2010), and my study (Chapter 5) are consistent with a selective role of latitude, mean temperature and temperature range. It is very interesting that I also found a positive correlation between yearly precipitation and the ratios of alkanes to all other tested substance classes. This is however not in line with the expectation to have a better protection against dessication in regions with lower precipitation and is opposite to the findings of Menzel et al. (2017). In addition, due to their physiological properties and water-proofing capabilities, CHCs appear to be particularly affected by humidity (Gibbs and Pomonis 1995, Gibbs 1998; Frentiu and Chenoweth 2010). However, I did not find significant correlations between CHC composition and humidity (Chapter 5), nor did Menzel et al. (2017). Overall, environmental factors seem to play a role in the evolution of CHCs in *Nasonia*.

**Intraspecific geographic variation of mating signals**

We also need a better understanding of local variation in mating signals within and among populations of a species, as this may point towards traits that are also involved in interspecific mate discrimination. This can also offer unique insights into the interplay between genes and the environment in the ontogeny of population differences (Miller et al. 1998; Bordenstein et al. 2000; Lachlan and Servedio 2004). Due to its wide geographical distribution, intraspecific geographical variation can be investigated in the *Nasonia* genus. For instance, intraspecific variation was reported in sexual isolation within and between two species of *Nasonia*, *N. vitripennis* and *N. longicornis* (Bordenstein et al. 2000), but the causes for this variation remained unknown. As geographical variation in mating behaviour and chemical signals of *Nasonia* remained to be investigated, I looked for latitudinal geographical differences of mating behaviour and chemical signals in *N. vitripennis*. Samples were collected at seven sites in Europe along a latitudinal cline from Corsica (42°N) to Northern Finland Oulu (65°N) with a distance between localities of 4-5 latitudinal degrees (Chapter 3). I performed several parallel studies with the same wasp populations to record male courtship behaviour courtship song, and female and male CHCs. My results revealed intraspecific quantitative differences between populations/strains in the number and duration of elements in male courtship components (Chapter 3), male courtship song patterns such as song bursts, pause durations and frequency (Chapter 4), and female and male CHC profiles (Chapter 5). Despite this, no difference in intra-population versus inter-population mate discrimination in terms of copulation success was evident (Chapter 3), suggesting that *N. vitripennis* in Europe can be considered as a single, rather undifferentiated, species. My results are in line with Paolucci et al. (2013) who found little
genetic differentiation ($F_{ST}$ values) between these same populations. Raychoudhury et al. (2010b) also reported low genetic variation in $N. vitripennis$ throughout Europe. Overall, European $N. vitripennis$ populations appear to be large enough to prevent significant differentiation by drift, and gene flow between subpopulations appears to be sufficient to prevent the build-up of reproductive isolation. Notably, I observed that the Turku populations differed somewhat stronger in male courtship behaviour (Chapter 3), female CHCs (Chapter 5), and song frequency (Chapter 4) than the other populations. The same Turku strains were found to differ more in photoperiodic diapause induction (Paolucci et al. 2013) and genetic marker composition (Paolucci 2014), which make these populations good candidates for further genetic studies of reproductive isolation.

I found modest but significant linear relations with latitude for some of the male courtship components (e.g., courtship initiation speed, latency time and headnod numbers in the first and second cycle within a courtship bout) (Chapter 3) and song patterns (e.g., burst length, pause duration between two bursts, and song frequency) (Chapter 4). These geographic patterns in courtship behaviour and courtship song may be coincidence, but are also consistent with our expectation that the environment exerts selection on some of the genes that underlie mating behaviour, in particular when these genes have pleitropic effects on other traits. For example, the clock genes $period$ was found to show a latitudinal cline in allelic composition (Paolucci et al. 2016). Clock genes are known to affect mating behaviour in insects (Ceriani et al. 2002; Reppert and Weaver 2002; Stoleru et al. 2004; Sandrelli et al. 2008; Allada et al. 2010; Stern 2014), such as courtship pulse song in $Drosophila$ (Kyriacou et al. 2017). Of course, at this point the link between clock genes and behaviour is still speculative, and more detailed functional studies are needed such as, for example, by silencing these genes with RNAi or CRISPR/Cas9 genome editing. No clear clinal pattern was observed in the full CHC profiles of females and males (Chapter 5). However, significant correlations were found in females, but not males, between some CHC ratios (e.g. alkanes - monomethyl-alkanes) / (alkanes + monomethyl-alkanes) with latitude and several climatic factors, such as mean temperature and temperature range among females (Chapter 5). Similar to clock genes, CHC components may be selected for adaptation to photoperiodic conditions depending on latitude and also affect aspects of courtship behaviour. Thus, the genetic architecture of prezygotic reproductive isolation in the $Nasonia$ genus is potentially complex and subject to multiple and opposing selection pressures.

**GENETIC BASIS OF MATING SIGNALS IN NASONIA**

Our knowledge of the genetic basis of reproductive isolation is still limited (Arbuthnott 2009; Presgraves and Glor 2010; Marie Curie SPECIATION network 2012; Giesbers et al.
One major focus in reproductive isolation is to identify the chromosomal regions and genes that reduce hybridization and gene flow. Insects such as *Nasonia* are a good model for investigating the reproductive isolation mechanisms. In *Nasonia*, differences in courtship behaviour and sex pheromones appear to be responsible for premating isolation between species.

Several studies on *Nasonia* have identified chromosomal regions associated with male courtship components and female mate discrimination (Velthuis *et al.* 2005; Peire Morais 2007; Niehuis *et al.* 2011; Gadau *et al.* 2012; Diao *et al.* 2016; Giesbers 2016). Fourteen QTL for male courtship behaviour in interspecific crosses between *N. vitripennis* and *N. longicornis* were found (J. Gadau *et al.* unpublished data). Velthuis *et al.* (2005) reported three major recessive loci for female mate choice between *N. vitripennis* and *N. longicornis*. Major QTL on chromosome 1, 2, 3 and 4 were found in interspecific crosses of *N. giraulti* and *N. oneida* when confronted with *N. vitripennis* males (Giesbers 2016). In addition, Niehuis *et al.* (2013) has narrowed down the genetic basis of production of the extra pheromone component *RR*-HDL in *N. vitripennis* to two genomic regions, one on chromosome 1 and one on chromosome 4. However, the underlying genes of mating behaviour and the genetic basis of pheromone quantity of *RS*-HDL have not yet been identified, despite the availability of full genome sequences of all *Nasonia* species (Werren *et al.* 2010).

In my QTL analysis (Chapter 6), I performed reciprocal interspecific crosses between the *N. giraulti* - *N. oneida* species pair combined with SNP markers in candidate genes aiming to determine simultaneously the genetic architecture of quantitative differences in male courtship behaviour, female preference and the long-range male sex pheromone *RS*-HDL. My results indicated that the *RS*-HDL quantity affects female receptivity in the mating process (Chapter 6). For male courtship components, I detected one QTL for cross direction (*i.e.*, which sex passed the border of the two joint glass tubes first) on chromosome 4 in crosses with *N. oneida* females, two QTLs for copulation success on chromosome 1 and 3 in crosses with *N. giraulti* females, but no QTL for other components of male courtship, such as number of cycles (Chapter 6). I detected four QTL for male pheromone quantity in *Nasonia*: one QTL on chromosome 4 in both the crosses with *N. giraulti* and *N. oneida* females, and this may be the same QTL as their positions overlap; another QTL on chromosome 1 in crosses with *N. giraulti* females only; one additional QTL on chromosome 5 is yielded in crosses with *N. oneida* females (Chapter 6). Whether our observed QTL of *RS*-HDL pheromone quantity on chromosomes 1 and 4 coincide with QTL of *RR*-HDL pheromone quality reported by Niehuis *et al.* (2013) requires a higher resolution QTL study.

Despite the large size of the mapping population, no significant QTLs were found for female preference in my study (Chapter 6), which points at a polygenic architecture of
female interspecific discrimination (i.e., with many small effect loci that went undetected because of insufficient power) with strong environmental influences. My results together with other studies on QTL in female mate discrimination (Velthuis et al. 2005; Giesbers 2016) demonstrated that the genetic architecture for mate discrimination in the Nasonia species complex consists of loci with major effects, and loci with minor effects, and differs to some degree based on the species pair considered.

Candidate genes known from mating behavior studies in Drosophila, such as protein-1-like, disco, lateNAy, and Ato on chromosome 4 were associated with the QTL for cross direction, and e, fru, fix_nod, XP001602953, per, dNA, cycle, lateNAy, csp, Mpk2(2), and Rhodophilin(B) on chromosome 1, and TipE, nonA, dco, beethoven, headnod, and acyl-CoA on chromosome 3 are associated with the QTL for copulation success (Chapter 6). Candidate genes, e, fru, csp, Mpk2(2), and Rhodophilin(B) on chromosome 1, protein-1-like, disco, lateNAy, Ato, and So(1) on chromosome 4, and slo, Fmr1, and Dy(2) on chromosome 5 are found to associate with QTL for RS-HDL pheromone quantity (Chapter 6). As pheromone quantity was shown to correlate with mating success, these genes are also candidate genes for mate discrimination in Nasonia. To further determine the possible role of the candidate genes in male courtship behaviour, RS-HDL quantity, and interspecific mate discrimination, more detailed functional analyses are needed, such as gene knock down studies.

CONCLUSIONS
For many years we have known that prezygotic isolation appears to evolve faster and at lower levels of overall genetic divergence than postzygotic isolation. Nevertheless, the genetic architecture of prezygotic reproductive isolation appears to be complex and subject to multiple and opposing selection pressures. The Nasonia genus is very suitable for studies of speciation, especially for the genetic changes that cause prezygotic reproductive isolation. Both inter- and intra-species variations exist in several mating signals in Nasonia, including male courtship behaviour, male courtship song, sex pheromone quantity and cuticular hydrocarbon composition, as described in this thesis. None of these mating signals, however, seem to act as efficient mating barriers by themselves, but together they cause different levels of prezygotic isolation between species pairs. Further functional investigations of these various traits are required to determine their exact role in sexual isolation. QTL studies on hybridizing species have been very informative about the underlying genetic architecture of the speciation process. Male courtship behaviour and pheromone quantity were demonstrated to have a genetic basis, and female interspecific mate discrimination is partly governed by these male traits, although in a complex way probably involving many genes of small effect and strong environmental effects. For further
progress in the understanding of the genetics of prezygotic reproductive isolation, more detailed genomic studies and functional knockdowns of candidate genes can be made within this model system.