Chapter 1 General introduction and thesis overview

Wenwen Diao

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SPECIATION
How new species arise, known as the process of speciation, is one of the most challenging questions of evolutionary biology because most of currently existing species arose thousands to millions years ago and are still evolving now. As changes in the phenotype only have evolutionary significance when they result from changes in the genotype that can be transmitted to successive generations, revealing which genetic changes lead to the origin of new species is important to understand Darwinian evolution.

In nature, there are four geographic modes of speciation, allopatric, peripatric, parapatric and sympatric, based on the extent to which speciating populations are geographically isolated from one another. Allopatric speciation, also known as geographic speciation, is speciation resulting from divergent evolution of populations that are geographically isolated from each other. In peripatric speciation, small peripheral populations are formed in isolation since small populations often undergo bottlenecks and can adapt rapidly to new niches. Populations in both allopatric and peripatric conditions can undergo genotypic and/or phenotypic divergence because of adaptation to different habitats (natural selection) or random genetic processes (bottleneck, genetic drift). Because of the genetic divergence in the absence of gene flow, when the populations come back into contact, they may no longer be capable of exchanging genes and have become reproductive isolated. Parapatric speciation is speciation resulting from divergent evolution of populations that are geographically adjacent to each other. The zones of two diverging populations are largely separate but do overlap to some extent. Populations under parapatric conditions can act as a source of divergence by natural selection as they reduce the fitness of matings between populations leading to selection for behaviours or mechanisms that prevent interbreeding. Sympatric speciation is the formation of two or more descendant species from a single ancestral species all occupying the same geographic location (Coyne and Orr 2004). It has long been debated how such populations can diverge while they share the same habitat and potentially exchange genes (Mayr 1963; Futuyma and Mayer 1980; Bush 1994; Orr and Smith 1998; Coyne and Orr 2004; Marie Curie SPECIATION network 2012). However, some compelling examples have been documented, e.g., host race formation in insects such as the apple maggot fly (Feder et al. 1995, 2003, 2008; Drès and Mallet 2002; Olsson et al. 2009; Powell et al. 2014). Although each mechanism may have different relative importance in driving biodiversity, all these forms of natural speciation have taken place over the course of evolution (Baker 2005).

REPRODUCTIVE ISOLATION
Under the biological species concept species are defined as independent reproductive entities, i.e., two individuals that cannot produce viable and fertile offspring are considered
belonging to different species. One important question in speciation biology is how reproductive isolation barriers become established.

There are two general types of reproductive barriers, those that act before fertilization are called prezygotic isolation barriers and those that act after fertilization are called postzygotic isolation barriers (Orr and Presgraves 2000). Prezygotic isolation includes temporal isolation, ecological isolation, behavioural isolation and gametic incompatibility. Temporal isolation occurs when individuals do not mate because they are active at different times of day or in different seasons. Ecological isolation refers to individuals that mate in their preferred habitat, and therefore do not meet individuals of other species with different ecological preferences. Behavioural isolation means that potential mates meet, but choose members of their own species. Mechanical isolation occurs if copulation is attempted, but transfer of sperm does not take place. Gametic incompatibility is a late-acting form of prezygotic isolation and means that sperm transfer takes place, but the egg is not fertilized subsequently. Postzygotic isolation occurs after fertilization and refers to factors that reduce hybrid fitness as a consequence of genetic incompatibilities. It can be manifested as embryo inviability, hybrid inviability and sterility, or hybrid breakdown which is often the result of changes induced by neutral processes such as drift. Zygotic mortality occurs if the egg is fertilized, but the zygote does not develop. Hybrid sterility is when the hybrid is viable, but the resulting adult is sterile. Hybrid breakdown means that first generation hybrids are viable and fertile, whereas subsequent hybrid generations are inviable or sterile (Coyne and Orr 2004). Postzygotic isolation barriers are often the result of diverged genetic constitutions of species, but infectious bacteria are now also widely accepted as a speciation force (Coyne 1992; Bordenstein et al. 2001).

One important aspect of reproductive barriers is how they become genetically established. Genetic changes must somehow lead to reproductive incompatibilities, such as incompatible mating signals (e.g., sender-receiver interactions) that prevent interbreeding, incompatible ecological adaptations that when combined in hybrids render them unfit in either parental habitat, or incompatible gene interactions that cause intrinsic hybrid dysfunctions (e.g., hybrid inviability or sterility) (Presgraves 2010). Prezygotic isolation seems to evolve at lower levels of overall genetic divergence than postzygotic isolation, at least in sympatry (Coyne and Orr 1989). 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). Genetics of behaviour is therefore an important field of research for understanding the speciation process.

Unresolved issues related to reproductive isolation 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 changes cause reproductive isolation or come into being after the speciation event (Barton and Gale 1993; Rice and Hostert 1993; Nosil 2008; Marie Curie SPECIATION network 2012). Such knowledge is required to understand how selection and drift may cause changes in reproductive isolation between populations in the course of evolution. Given recent high-throughput sequencing and computational advances that allow genome-wide analyses, we are now capable of rapidly scanning large portions of the genome of both model and non-model organisms for differentiation to explore the genomic perspective of speciation (Nosil and Feder 2012).

Nevertheless, our knowledge of the genetic basis of reproductive isolation is still limited (Otte and Endler 1989; Coyne 1992; Coyne and Orr 1998; MacDonald and Goldstein 1999; Presgraves et al. 2003; Arbuthnott 2009; Beukeboom et al. 2010; Presgraves and Glor 2010; Marie Curie SPECIATION network 2012; Giesbers et al. 2013). One longstanding question is whether there exist “speciation genes”, e.g., whether certain genes are more often involved in causing reproductive isolation than others, or whether any gene can cause this. Speciation genes can be associated with any form of reproductive isolation barriers, but most is known about those related to hybrid dysfunction causing postzygotic isolation (Orr 2005; Presgraves and Glor 2010). Incompatibility between nuclear and mitochondrial genes are well known to be associated with reproductive isolation in plants (Mayr 1986), but plays a role in animals as well (Ellison et al. 2008; Gibson et al. 2010). Presgraves (2010) and Johnson (2010) have argued that genetic changes causing hybrid incompatibility might be fixed for non-adaptive reasons, for example, coevolution of meiotic drivers and their suppressors.

Even less is known about the genetic basis of traits responsible for prezygotic isolation (Arbuthnott 2009). For instance, we still know little about the genetic basis of behavioural differences between species and how easily such behaviours can change (Ritchie and Philips 1998; Shaw and Parsons 2002; Arbuthnott 2009; Laturney and Moehring 2012; Giesbers et al. 2013; Niehuis et al. 2013; Diao et al. 2016). Some studies have identified key genes, especially for chemical signalling (Shirangi et al. 2009; Smadja and Butlin 2009; Lassance et al. 2010), but in many cases analyses remain at the quantitative trait locus (QTL) level (e.g., Gleason and Ritchie 2004; Shaw et al. 2007; Laturney and Moehring 2012). Genomic and functional genetic studies (e.g., knock down of candidate genes) should further document the molecular basis of isolating traits that drive divergence (Marie Curie SPECIATION network 2012).

In this thesis, I focus on the genetics and genomics of prezygotic reproductive isolation in *Nasonia* wasps that have become a model system for evolutionary biology. I investigate phenotypic and geographical variation of traits which may influence prezygotic isolation,
such as mating behaviour and chemical communication signals. I perform a QTL study combined with markers in candidate genes to determine the genetic architecture of mating behaviour and sex pheromones. The results of this research are expected to contribute to the knowledge of the genetic basis of reproductive isolation in speciation.

THE NASONIA GENUS - A MODEL SYSTEM FOR SPECIATION STUDIES

Nasonia (Hymenoptera, Pteromalidae) are 2-3 mm sized parasitoid wasps that sting and lay eggs in pupae of cyclorrhaphous flies, such as Calliphora and Protocalliphora, which are found in bird nests and on carcasses (Whiting 1967; Pultz and Leaf 2003; Grillenberger et al. 2008). After egg hatching, the fly larvae feed by sucking blood of nestlings. They usually pupate shortly after nestlings have fledged and this is the developmental stage that flies are parasited by Nasonia. A wasp’s clutch typically consists of 20-30 eggs depending on host size (Charnoy and Skinner 1984). Offspring typically mate on the patch after which females disperse in search of new host patches. Females typically mate only once but they may become receptive a second time, in particular in laboratory cultures (van den Assem and Visser 1976; Grillenberger et al. 2009). Males can mate multiple times with their female partners.

The Nasonia genus contains four closely related species: N. vitripennis (Walker 1836), N. longicornis, N. giraulti (Darling and Werren 1990) and the more recently discovered N. oneida (Raychoudhury et al. 2010a). N. vitripennis can be found throughout the world, but the other three species only occur in North America, where their ranges partially overlap (see geographical distribution in Figure 1.1). Phylogenetic analyses (see Figure 1.1) revealed that N. vitripennis was the first species that split from its sister species, and N. oneida is the youngest species that split recently from N. giraulti (Campbell et al. 1993; Raychoudhury et al. 2010a, 2010b).
Figure 1.1: Geographical distribution and phylogenetic relationship (with Trichomalopsis sarcophagae as outgroup) of the Nasonia genus. The figure of geographical distribution is taken from Giesbers et al. (2013). The figure of the phylogenetic tree is adapted from Werren et al. (2010) with data of Raychoudhury et al. (2010a).

The Nasonia genus has been used extensively for research on the genetics of speciation and species differences (Breeuwer and Werren 1990, 1993, 1995; Gadau et al. 1999, 2000, 2002; Bordenstein et al. 2000, 2001, 2003; Beukeboom and van den Assem 2001, 2002; Velthuis et al. 2005; Niehuis et al. 2008, 2010, 2013; Werren and Loehlin 2009; Loehlin et al. 2010a, 2010b; Werren et al. 2010; Buellesbach et al. 2013). There are several advantages of Nasonia for genetic study of reproductive isolation barriers. One is its haplodiploid reproduction: males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs. As dominance and recessive effects do not exist in haploids, haplodiploidy greatly facilitates quantitative genetic analysis of traits in males, such as genetic linkage mapping and QTL studies (Gadau et al. 1999, 2002; Koevoets and Beukeboom 2009; Loehlin et al. 2010a, 2010b; Niehuis et al. 2011; Gadau et al. 2012). Another advantage is the feasibility of interspecific crosses in the laboratory. In nature, the four Nasonia species are reproductively isolated due to infection with species-specific strains of Wolbachia bacteria that cause strong postzygotic isolation through cytoplasmic incompatibility and hybrid breakdown in interspecific crosses (Breeuwer and Werren 1990, 1995; Bordenstein and Werren 1998; Bordenstein et al. 2001). Antibiotic (e.g., tetracycline) curing in the laboratory allows interspecific crosses and genetic analysis of species-specific traits (Breeuwer and Werren 1990, 1993; Werren and Loehlin 2009; Sharon et al. 2011). Two Wolbachia-free species can successfully produce fertile hybrids, allowing gene mapping. Other advantages of the Nasonia species complex are the availability of full genome sequences of the four species, and high density marker maps (Werren et al. 2010). This makes positional cloning of candidate genes identified with QTL studies feasible, as recently demonstrated for a wing size difference (Loehlin et al. 2010a, 2010b), and a pheromone component polymorphism (Niehuis et al. 2013).
The laboratory culturing of *Nasonia* is simple. They take up little space and readily mate and reproduce in the laboratory environment. Generation time is only 2 weeks (at 25°C). Wasps are immobile in the pupal stage and therefore can be collected by breaking open the fly hosts without the need for anesthetization. Individuals are most easily sexed in the black pupal stage after minimal training. The easiest way to distinguish them is by looking for the presence of an ovipositor in the distal end of the abdomen. In *N. vitripennis*, males can also be distinguished by small wing pads. Furthermore, *Nasonia* can be easily obtained from the field (Whiting 1967; Grillenberger *et al.* 2008; Paolucci *et al.* 2013). Wasps can be collected from bird nests as they parasite and develop in fly pupae that occur in bird nests. The time with high chance of collecting *Nasonia* is just after birds have fledged. Nests can be removed and dissected to look for parasitized fly pupae. Alternatively, adults *Nasonia* can also be collected directly in the bird nests or baits that contain fly pupae. After collection, isofemale strains can be established either from the adults collected directly from the field or from progenies that emerged from pupae of bird nests or baits.

*Nasonia* has a facultative diapause that occurs at the fourth larval instar just before pupation and it is induced by the mother depending on photoperiod and age (Saunders 1965; Paolucci *et al.* 2013). Diapause larvae can be kept under diapause condition (5°C, 0:24 h light/dark cycle) in the laboratory for more than a year. This means their maintenance efforts can be reduced which is very convenient to keep field-collected lines alive. A schematic life cycle of *Nasonia* is shown in Figure 1.2.

![Figure 1.2](image-url): life cycle of *Nasonia* consisting of several developmental stages. Photos were taken by Peter Koomen.
PREZYGOTIC ISOLATION IN NASONIA

In the *Nasonia* species complex, all species perform a complex mating ritual which consists of a series of interactions between the male and female and ends with female receptivity and copulation (Whiting 1967; van den Assem 1986; van den Assem and Werren 1994; Beukeboom and van den Assem 2002; Velthuis *et al.* 2005; Burton-Chellew *et al.* 2007). The *Nasonia* male releases sex pheromones, perform courtship behaviours and produce sounds by vibrating their wings to induce female receptivity (van den Assem *et al.* 1980; van den Assem 1986; Clark *et al.* 2010). Given that there is clear interspecific female mate discrimination between *Nasonia* species (Raychoudhury *et al.* 2010a; Giesbers *et al.* 2013) and that some species occur in sympatry and others in allopatry, the importance of these traits in causing prezygotic isolation can be well investigated.

Courtship behaviour is known to play a pivotal role in the onset of population divergence leading to premating reproductive isolation between species (e.g., Hoikkala *et al.* 2000; Williams *et al.* 2001; Gleason *et al.* 2002; Gleason and Ritchie 2004; Mackay *et al.* 2005; Arbuthnott 2009; Joyce *et al.* 2010; Giesbers *et al.* 2013). The *Nasonia* species differ in male courtship behaviour and can be recognized by their species-specific courtship pattern with both qualitative and quantitative aspects (van den Assem and Werren 1994; Drapeau and Werren 1999). Courtship and mating behaviour have been well described in three of the *Nasonia* species, *N. vitripennis*, *N. longicornis* and *N. giraulti* (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). However, the complete characteristics of male courtship behaviour of the youngest species in the genus, *N. oneida*, have not been described in full. The role of differences in male courtship behaviour in preventing interspecific mating is not clear. For example, it is not known to what extent the differences in male courtship behaviour are perceived by the female 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. As to the genetic basis of courtship behaviour, several chromosomal regions that are associated with specific male courtship components and female mate discrimination have been identified (Velthuis *et al.* 2005; Peire Morais 2007), but, the underlying genes of these behaviours have not yet been characterized.

Male courtship song, created by wing vibration, is another factor which may play a role in mate choice. Song has been shown to be an important part of male courtship behaviour in many species of *Drosophila* (e.g., Cowling and Burnet 1981; Kyriacou and Hall 1982; Cobb *et al.* 1989; Ritchie and Kyriaco 1994; Ritchie and Gleason 1995, Demetriades *et al.* 1999; Saarikettu *et al.* 2005; Hoikkala *et al.* 2007; Veltsos *et al.* 2012; Debelle *et al.* 2014). In
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*Nasonia*, male wasps also vibrate their wings (van den Assem 1986) as part of their courtship behavior, but the role of song differences in inducing female receptivity is unknown. Van den Assem and Putters (1980) provided a more detailed description of *N. vitripennis* sonograms: a continuous repetition of a horseshoe like pattern, at about 3 per 2 seconds (at 22°C), with the start and end at about 50 Hz, and a peak frequency of about 400 Hz. The song characteristics of the other three *Nasonia* species have not been reported and any inter- and intra-specific variation remains to be analyzed. Moreover, no study has addressed the genetic architecture of courtship song in *Nasonia*.

Chemical signals are probably the oldest means of communication in insects. Volatile sex pheromones are often produced by one of the two sexes to form a specific odour that is recognized by the other sex leading to attraction and copulation between the partners. *Nasonia* males can produce long-range and short-range sex pheromones to attract the female from distance (van den Assem et al. 1980; Ruther et al. 2007, 2008) and to induce receptivity in courted females (van den Assem et al. 1980; Ruther et al. 2010). The long-range sex pheromone is produced from the gland of male’s abdomen and consists of three components (4R,5R)-5-hydroxy-4-decanolide (RR-HDL), (4R,5S)-5-hydroxy-4-decanolide (RS-HDL) and 4-methylquinazoline (Ruther et al. 2007, 2008). Niehuis et al. (2013) reported that *N. vitripennis* is the only *Nasonia* species whose males biosynthesize the long-range sex pheromone component RR-HDL, besides RS-HDL and 4-methylquinazoline. The genetic basis of production of the extra chemical component RR-HDL in *N. vitripennis* has been narrowed down by genetic mapping to two genomic regions, one on chromosome 1 and one on chromosome 4 (Niehuis et al. 2013). Yet, the quantity of long-range sex pheromone (e.g., RS-HDL), which might determine the duration of the latency time, has not been investigated. The short-range pheromone is deposited by mandible contact of the male during courtship on the female’s antennae to induce female receptivity (van den Assem et al. 1980), but its chemical composition has to date not been elucidated.

In addition to volatile sex pheromones, non-volatile pheromones such as cuticular hydrocarbons (CHCs) present on the body surface of insects can also act as chemical cues in mate choice. They serve multiple functions, including protection against desiccation, barrier against microbial infection, recognition cues, and sex pheromones (Edney 1977; Hadley 1981; Coyne et al. 1994; Wagner et al. 2001; Ferveur 2005; Howard and Blomquist 2005; Lucas et al. 2005; Mas and Jallon 2005; Johansson and Jones 2007; Peterson et al. 2007; Blomquist and Bagneres 2010; Veletsos et al. 2012; Chung and Carroll 2015; Wurdack et al. 2015). Buellesbach et al. (2013) reported that in all *Nasonia* species except *N. giraulti* the CHC profiles of the female are a cue for males to decide whether or not to mount the female as part of the mating behavior repertoire. Raychoudhury et al. (2010a) and Buellesbach et al. (2013) found that CHC profiles of the *Nasonia* species differ strongly.
Niehuis et al. (2011) identified genes for alkene biosynthesis with a high similarity to *Drosophila* in a QTL study of cuticular hydrocarbon differences between *N. giraulti* and *N. vitripennis*. No studies have considered the potential influence of geoclimatic factors on insect CHCs composition.

Divergence of mating signals and mate recognition systems is considered as one of the most important factors in speciation (Shaw and Parsons 2002). Analysis of local variation within and among populations of a species may reveal phenotypic differences that affect mate choice and gene exchange. This can offer unique insights into the interplay between genes and environment in the ontogeny of population differences (Miller et al. 1998; Bordenstein et al. 2000; Lachlan and Servedio 2004). In general, genetic differentiation increases with geographical distance between populations (Mayr 1963; Coyne and Orr 2004). Genetic divergence arises from selection for adaptations to local environments among allopatric populations (Coyne and Orr 1998). In addition, genetic drift may lead to differences in behaviour and sexual isolation when such divergence is correlated with behaviour (Mayr 1963; Koepfer 1987; Coyne and Orr 2004). Due to its wide geographical distribution and well-studied mating behaviour, geographical variation in reproductive isolation can be investigated in *Nasonia*. Moreover, the environment may exert selection on some of the genes that are coding for courtship behaviour and CHCs, in particular when these genes have pleitropic effects on other traits. For example, clock genes may be selected for adaptation to photoperiodic conditions depending on latitude and also affect aspects of courtship behaviour, or genes that underlie CHC profiles may undergo selection for desiccation resistance depending on climatic conditions as well as for mate recognition. Thus, the genetic architecture of reproductive isolation is potentially complex and subject to multiple and opposing selection pressures.

**OUTLINE OF THIS THESIS**

This PhD research is part of the EU funded Marie Curie Initial Training Network (ITN) “SPECIATION”, with researchers from four European universities; University of Sheffield (England, UK), University of St. Andrews (Scotland, UK), University of Jyväskylä (Finland) and University of Groningen (The Netherlands). The aim of the “SPECIATION” network is to develop nine PhD projects under three inter-related research themes: (1) the genetics and genomics of reproductive isolation, (2) behavioural mechanisms of speciation, (3) evolutionary and ecological drivers of diversification, in order to understand the evolutionary origin of biological diversity and to study mechanisms of speciation. The approach is to select powerful model systems with which to investigate the progress of speciation and related mechanisms, and to apply modern techniques from quantitative and behavioural genetics, molecular ecology and environmental genomics.
The goal of my PhD research project is to investigate phenotypic variation and genetic architecture of traits underlying prezygotic reproductive isolation in the *Nasonia* species complex: mating behaviour (including courtship behaviour and courtship song) and chemical communication (including cuticular hydrocarbons and sex pheromones). I aim to mainly answer the following questions: What is the variation in potential prezygotic isolation traits within and between species? Is there any latitudinal geographic difference of those traits within a species? What is the genetic basis of mating behaviour and sex pheromone variation? By addressing these questions, I aim to answer whether mating behaviour and chemical signals act as efficient prezygotic isolation barriers in reproductive isolation during the process of speciation.

In **Chapter 2**, I first investigate one potential prezygotic isolation trait - courtship behaviour - of the youngest species in the *Nasonia* genus, *Nasonia oneida*, to complete the description of male courtship behaviours in the *Nasonia* species complex. Another goal of this chapter is to evaluate whether this species can be used in experiments aimed at identifying the genetic basis of prezygotic reproductive isolation (see Chapter 6). My results demonstrate that *N. oneida*, like the other *Nasonia* species, can also be recognized by its species-specific courtship characteristics and that it resembles most *N. giraulti*. Given the strong behavioural isolation of *N. oneida* from the other *Nasonia* species, this species was identified as very suitable for further genetic analysis of prezygotic isolation factors within the *Nasonia* genus.

In **Chapter 3**, I study the phenotypic intraspecific variation of male courtship behaviour and female choice of the widespread species, *Nasonia vitripennis*, collected in Europe along a latitudinal cline from Southern Italy to Northern Finland. Additionally, I analyse the correlation between behavioural variation and latitude. My results reveal variation in some of the male courtship components (e.g., number and duration of elements) among populations, but only few behavioural components followed a latitudinal gradient (including courtship initiation speed, latency time and headnod numbers in the first and second cycle within a courtship bout). No difference in intra-population versus inter-population mate discrimination was found. All results suggest that the populations are large enough to prevent detectable drift effects on geographical differentiation in courtship and mating behaviour in this species.

In **Chapter 4**, I investigate interspecific and intraspecific phenotypic variation of another prezygotic isolation trait – courtship song, created by wing vibration during male courtship. Laboratory strains of all four *Nasonia* species and field strains of European *Nasonia vitripennis* were used for this study. My results indicate that all *Nasonia* species share a clear hum song that is similar to the sine song in *Drosophila*, but they do not have a pulse song. Species-specific male courtship songs were evident in *Nasonia*. Variation was
detected in the song patterns (including song bursts, pause durations and frequency) among *N. vitripennis* strains, and this variation correlated weakly with latitude. The overall pattern of the song shows similarity to behavioural components of courtship.

In **Chapter 5**, I identify intraspecific phenotypic variation of another prezygotic isolation trait – cuticular hydrocarbon (CHC) sex pheromones – in European *Nasonia vitripennis* strains. I compare CHC profiles with two American *N. vitripennis* strains, one *N. giraulti* and one *N. longicornis* strain. I correlate ratios of CHC substance classes, including alkanes, alkenes, monomethyl-alkanes, dimethyl-alkanes, and trimethyl-alkanes, with several geoclimatic factors, including latitude, mean temperature, temperature range, humidity and precipitation, to identify possible impact of geoclimatic factors on the genetic background of intraspecific CHC composition. I find that European *N. vitripennis* CHC profiles, in both female and male, overlap with those of American *N. vitripennis*, but are different from the other two species, which is consistent with species- and sex-specific CHC compositions of *Nasonia*. Intraspecific variation is found in CHC profiles of both females and males, and the variation is not along a latitudinal gradient. Significant correlations are found between the CHC ratio (alkanes - monomethyl-alkanes) / (alkanes + monomethyl-alkanes) with latitude, mean temperature, and temperature range among females. Furthermore, significant correlations are detected between CHC ratios in females, but not in males, with mean annual precipitation rates. These results suggest that geoclimatic factors have an impact on female CHC compositions in *N. vitripennis*.

In **Chapter 6**, I investigate the genetic architecture of male pheromone quantity, male courtship behaviour and female choice, as aspects of prezygotic isolation. I use the youngest species pair of the *Nasonia* species complex, *Nasonia giraulti* and *Nasonia oneida*, that exhibit strong prezygotic isolation. With reciprocal interspecific crosses between these two species, I designed a QTL study, involving 475 recombinant hybrid males (F$_2$) and 2148 reciprocally backcrossed females (F$_3$). With genome sequence information, a linkage map of 92 single nucleotide polymorphism (SNP) markers including 52 equally spaced neutral SNP markers plus SNPs in 40 candidate mating behaviour genes were used. My results demonstrate that the RS-HDL pheromone plays a role in the mating system of *N. giraulti* and *N. oneida*. I detect four QTL for male pheromone quantity. No QTL are found for female mate discrimination, which points at a polygenic architecture of female choice with strong environmental influences.

In **Chapter 7**, I synthesize all results of the previous chapters. I summarize the current knowledge of prezygotic reproductive isolation factors in *Nasonia* wasps and discuss what it means for our understanding of the process of speciation.
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